The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 43 MARCH, 1954 NO. 3

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

Determination of Gastric Secretory Function by Measurement of Substances Excreted by the Kidneys. I. Uropepsin Excretion in Health and Disease. Robert J. Bolt, M.D., H. Marvin Pollard, M.D., and Alvaro Carballo, M.D., Ann Arbor, Mich. 335

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Vol. 43, No. 3, March, 1954. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription price: United States and its Possessions $12.00 per year; Canada $13.00; Foreign $13.50. Subscriptions to medical students, interns and residents, half-price. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

The Journal of Laboratory and Clinical Medicine

VOL. 43 MARCH, 1954 NO. 3

CLINICAL AND EXPERIMENTAL

DETERMINATION OF GASTRIC SECRETORY FUNCTION BY MEASUREMENT OF SUBSTANCES EXCRETED BY THE KIDNEYS

I. UROPEPSIN EXCRETION IN HEALTH AND DISEASE

ROBERT J. BOLT, M.D., H. MARVIN POLLARD, M.D., AND ALVARO CARBALLO, M.D.
ANN ARBOR, MICH.

IN 1860 Brucke[1] first reported the presence of a pepsin-like enzyme in the urine. Loeper and Esmonet[2] were among the first workers to suggest that this urinary pepsin might be a proferment secreted directly into the blood stream by the peptic cells of the gastric mucosa. Like pepsin it is active in highly acid media, but unlike pepsin, it is stable in weak alkali, a physicochemical characteristic of pepsinogen, the precursor of pepsin. Mirsky and co-workers[3] have shown that feeding of pepsin does not produce an increase in uropepsin excretion. Intravenous administration of pepsin likewise failed to result in an increase in uropepsin excretion. Oral administration of pepsinogen (dog) similarly failed to result in increased excretion. However, when pepsinogen was administered intravenously increased uropepsin excretion resulted. From these experiments it would seem that the proteolytic enzyme found in urine is secreted by the gastric cells directly into the blood stream. Broh-Kahn, Podore, and Mirsky[4] postulated that the rate of excretion of uropepsin might be related to the intrinsic endocrine activity of the peptic cells. Their findings indicated that healthy men have a constant rate of excretion unaffected by time of night or day, volume, specific gravity, acidity, exercise, or fluctuations in dietary habits. The same group of workers[5] reported that duodenal ulcer patients excreted at an average rate approximately two times that of normal individuals. It was suggested that

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Received for publication, Oct. 13, 1953.

uropepsin excretion might be of help in the diagnosis of duodenal ulcer. Other reports have appeared in the literature showing variable and inconclusive results. Spiro, Reifenstein, and Gray[6] have shown an increased uropepsin output during the administration of adrenocorticotropic hormone. Farnsworth[7] reported the presence of uropepsin in reduced amounts in patients with pernicious anemia. Mirsky[8] reported a variation in uropepsin excretion with changes in emotional states. In gastric apepsia without carcinoma uropepsin has been reported by Okada[9] to be frequently absent but in none of 109 patients examined by Gottlieb[10] was it found to be absent. Bucher[11] reported a relatively constant output in patients on a uniform daily diet but a marked increase in uropepsin excretion was seen when the protein intake was doubled.

It has been suggested by certain investigators[5, 7, 12] that the measurement of uropepsin excretion might be used as a substitute for the standard gastric analysis in the evaluation of hyper- and hyposecretory states of the gastric mucosal cells. Janowitz[12] has likewise suggested that this test might be of value in the differential diagnosis of bleeding gastrointestinal lesions. In view of these possibilities we have undertaken the clinical evaluation of uropepsin excretion in normal patients as well as in those with various gastrointestinal lesions.

MATERIAL AND METHODS

Both hospital patients and college students were used as subjects. Separate analyses were done on 216 urine specimens collected from 51 subjects. These included 18 individuals without known gastrointestinal disease, 16 patients with duodenal ulcer, 5 patients with ulcerative colitis, 8 patients with gastric ulcer, and 4 with gastric carcinoma. Twelve hour urine specimens (8:00 P.M. to 8:00 A.M.) were collected under toluene. The volume was measured and a portion stored in the refrigerator. The urine was never stored more than fifteen days before analysis, although others have stated that there is no loss in uropepsin activity up to twenty-eight days when the urine is stored in this way. The method of analysis employed was exactly as outlined by G. R. Bucher.[11] There was always an average variation in repeated analysis on the same urine of about 50 mg. per twenty-four hours. Four separate nightly collections were made on each patient and the average twenty-four hour excretory rate was determined from the twelve hour volume and uropepsin concentration. Results were expressed as milligrams of "tyrosine" as recommended by Bucher.[11]

RESULTS

The results are depicted in Fig. 1. The first column comprises 18 patients without demonstrable gastrointestinal disease and without complaints referable to the gastrointestinal system. As can be seen there is a marked variation in rates of excretion among this control group. The lowest excretory rate is 98 mg. "tyrosine" per twenty-four hours. The maximum excretory rate is 835 mg. of "tyrosine" per twenty-four hours and the average is 417 mg. per twenty-four hours.

In the duodenal ulcer group the average twenty-four hour excretory rate is 810 milligrams of "tyrosine" or approximately two times the average rate for the control group. This agrees closely with results reported by other observers. The variation in the duodenal ulcer group, as in the control group, is extremely wide. The lowest rate is 390 mg. per twenty-four hours and the

highest rate 1,710 mg. The latter patient (S. K. No. 610,345) was shown to have a posterior penetrating duodenal bulb ulcer as well as a second ulcer in the prepyloric region. If the two groups (control and duodenal ulcer) are compared a great deal of overlap is apparent. The majority (83.3 per cent) of the control group had an excretory rate below 700 mg. per twenty-four hours while only 26.6 per cent of the duodenal ulcer group were below this level. Conversely only 16.7 per cent of the control group excreted at a rate greater than 700 mg. per twenty-four hours while 73.4 per cent of the ulcer group were well above this rate. The 8 patients with gastric ulcer and the 5 with ulcerative colitis had average excretory rates midway between that found in the control and duodenal ulcer groups. Again there is a marked spread when individuals in the same group are compared. Patients with proved gastric carcinoma had approximately the same excretory rate as those in the control group.

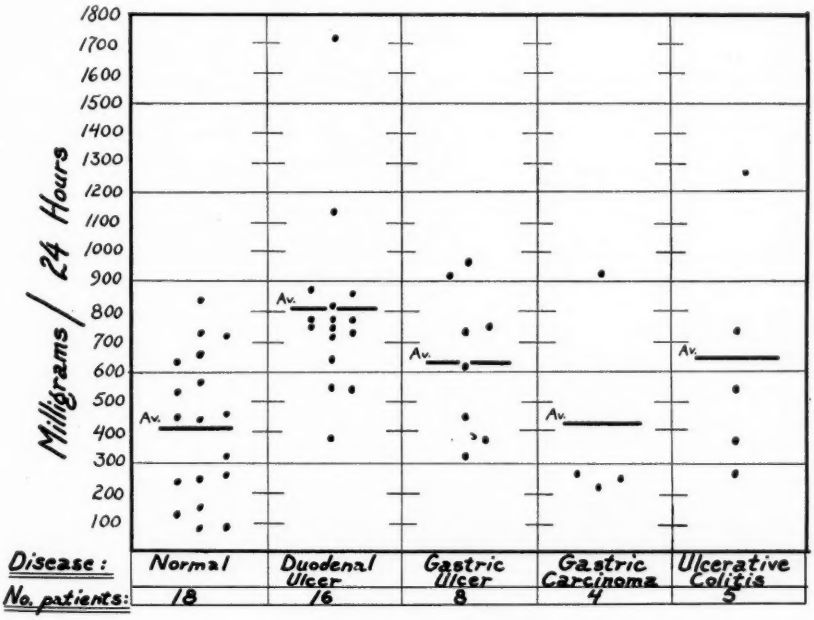

Fig. 1.—Twenty-four-hour uropepsin excretory rate.

DISCUSSION

Our results confirm the higher rate of uropepsin excretion seen in duodenal ulcer patients when the average rate of a number of subjects is compared with the average rate of a number of "nonulcer" individuals. The variation within each group was of sufficient magnitude to cause a rather marked overlap when comparing individual patients. The finding of a low rate of excretion in the individual patient does not therefore place that individual definitely in the nonulcer group. Conversely the individual with a high uropepsin output cannot be placed in the ulcer group with any degree of certainty. In our series a uropepsin

level above 700 mg. of "tyrosine" per twenty-four hours could be considered supportive but not diagnostic evidence of the presence of a peptic ulcer. In view of the necessity for collecting accurate specimens of urine over a measured period of time and the difficulty with which the analysis is carried out it is doubtful that this test could be utilized as a routine procedure in most general hospitals. Unfortunately the collection of a single specimen of urine and analysis for uropepsin concentration has been shown to be extremely variable and of no or little value in differential diagnosis. (Fig. 2.)

In the patients observed by us with benign gastric ulcer, gastric carcinoma, and ulcerative colitis there was little difference in either the rate (Fig. 1) or the concentration (Fig. 2) of urinary pepsinogen excretion.

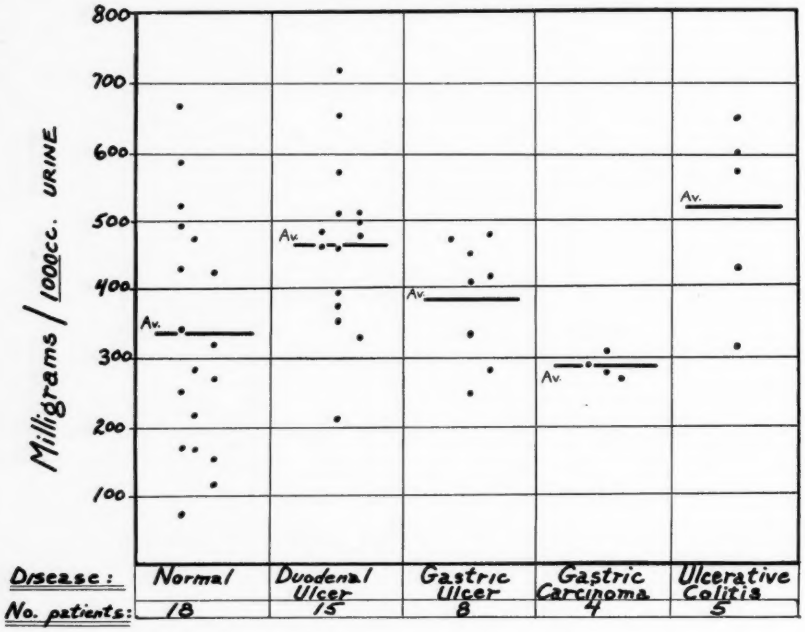

Fig. 2.—Uropepsin concentration.

CONCLUSIONS

Urinary pepsinogen is excreted by duodenal ulcer patients at an average rate of approximately two times that seen in normal individuals.

Urinary pepsinogen concentration, in contrast to rate of excretion, was only slightly higher in duodenal ulcer patients as compared to the normal group.

The marked variation in the pepsinogen excretory rate in each of our groups, including patients with duodenal ulcer, gastric ulcer, gastric carcinoma, and ulcerative colitis makes the test unreliable for differentiating between individual cases as to the presence or absence of a peptic ulcer.

The urinary pepsinogen excretory rate used in conjunction with the patient's history, physical examination, and other laboratory tests gives additional evidence for or against the presence of a peptic ulcer if the values are above 700 or below 300 mg., respectively.

The difficulty with which the test is performed and the necessity for accurate periods of urine collection militate against the urinary pepsinogen excretory rate being used as a routine diagnostic procedure.

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DETERMINATION OF GASTRIC SECRETORY FUNCTION BY MEASUREMENT OF SUBSTANCES EXCRETED BY THE KIDNEYS

II. An Evaluation of the Tubeless Method of Gastric Analysis

H. Marvin Pollard, M.D., Alvaro Carballo, M.D., and Robert J. Bolt, M.D.
Ann Arbor, Mich.

RECENTLY the utilization of the cation exchange indicator Diagnex,* has been suggested as a method for determining the presence or absence of free hydrochloric acid in gastric contents without the need for gastric intubation.[1, 2] Segal[3] has reported an excellent correlation between the tubeless method and the standard intubation technique for gastric analysis. Malach and Banks[4] subsequently reported on the results in 54 patients in whom no form of gastric gland stimulation was used. Their comparison with intubation likewise showed the Diagnex method to be highly efficient. There is a need for further amplification and verification of these studies. For this reason we undertook an evaluation of the quininium cation exchange indicator Diagnex as a measure of gastric secretory activity. This report is concerned with the results in 76 patients studied both by the tubeless and intubation techniques of gastric analysis.

RATIONALE

The reader is referred to articles by Segal[1-3] and Malach and Banks[4] for more complete statements regarding the basis for the development of this test. In the preparation of Diagnex the hydrogen cation of a carboxylic acid group in the exchange resin is replaced by a quininium cation. In the presence of free hydrochloric acid the hydrogen cation of the acid replaces the quininium cation releasing quinine. The quinine is absorbed in the small intestine and approximately one-third is excreted in the urine.[3] This test is then based on the quantitative estimation of quinine in the urine.

METHODS AND MATERIAL

In conducting the test the following procedure was carried out. The patient was given no food after midnight. The first urine specimen passed on arising was saved and marked "fasting." At 9:00 A.M. the patient was given one-half glass of water containing caffeine.† This was followed by another one-half glass of water. At 10:00 A.M. the patient was asked to void and the specimen discarded. Granules were then put into a glass and water added to approximately one-fourth glass.‡ It was stirred vigorously and given immediately to the

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Received for publication, Oct. 13, 1953.

*E. R. Squibb & Sons, New York.

†250 mg. of caffeine sodium benzoate.

‡Granules consist of 2 Gm. of quininium indicator compound, marketed under the trade name of Diagnex, and kindly supplied by E. R. Squibb & Sons, New York.

patient to drink. This was followed by another one-fourth glass of water. If any particles remained in the glass this water was used for further mixing. One and two hours, respectively, after taking the granules urine specimens were obtained and appropriately marked. The patient was instructed to empty the bladder completely each time. If the patient found it necessary to void before the specified time, he was instructed to save the urine and add it to the urine passed at the time scheduled. All patients included in this report were subjected to both the quininium test and the conventional gastric analysis. When the tube method was employed, histamine diphosphate (0.5 mg. of the base by subcutaneous injection) was employed as the stimulant. In making comparisons of the two methods the highest amount of free acid following histamine stimulation was correlated with the second hour urinary quinine concentration. The ether-sulfuric acid method of Kelsey and Geiling[5] was utilized in reading the results. The specimens tested were compared with standard solutions of quinine of the following strengths:

STANDARDS FOR INTERPRETATION

REPORT	CONTROL
Faint trace	0.005 mg. quinine
Trace	0.012 mg. quinine
One plus	0.040 mg. quinine
Two plus	0.076 mg. quinine
Three plus	0.145 mg. quinine
Four plus	0.180 mg. quinine

In this report both the first and second urine specimens were analyzed regardless of the results of the first urine specimen. In no case was less quinine found in the second specimen when compared with the first. In practice it would therefore seem to be necessary to test the second specimen only when the first urine specimen is negative or shows a faint trace of quinine. If the first hour specimen is negative or shows a faint trace of quinine the following combinations are possible:

SPECIMEN NO. 1	SPECIMEN NO. 2
Negative	Negative
Negative	Faint trace
Negative	Trace
Faint trace	Faint trace

The above combinations all signify the absence of free gastric hydrochloric acid and, for the sake of simplicity, are included as the negative-trace group of patients.

The following combinations signify the presence of free gastric hydrochloric acid and are reported as showing a one plus reaction:

SPECIMEN NO. 1	SPECIMEN NO. 2
Negative	One plus
Faint trace	Trace
Faint trace	One plus
One plus	One plus

In our series of patients the faint trace—trace combination did not happen to occur. Since this combination does signify the presence of free hydrochloric acid, it should be included in this group when it does occur.

In classifying our patients as two, three, or four plus reactors the second hour urine specimen was utilized since this was always the same as or, in most cases, higher in quinine concentraction than the first hour urine specimen.

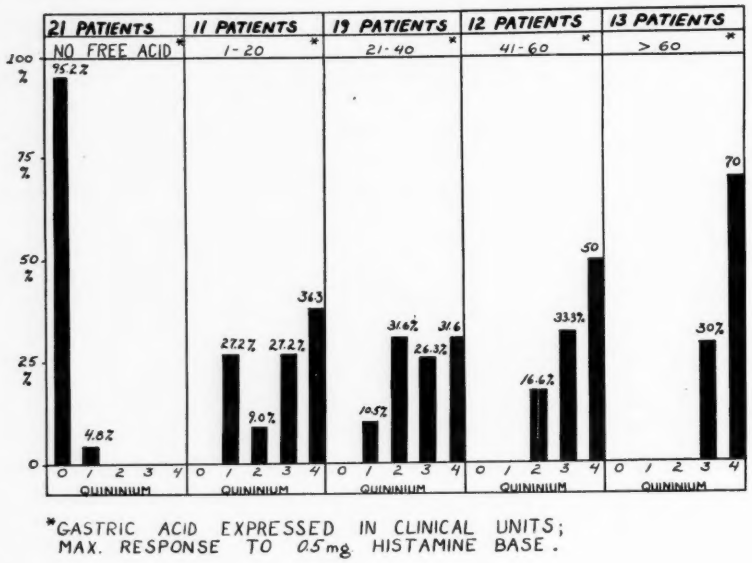


Fig. 1.

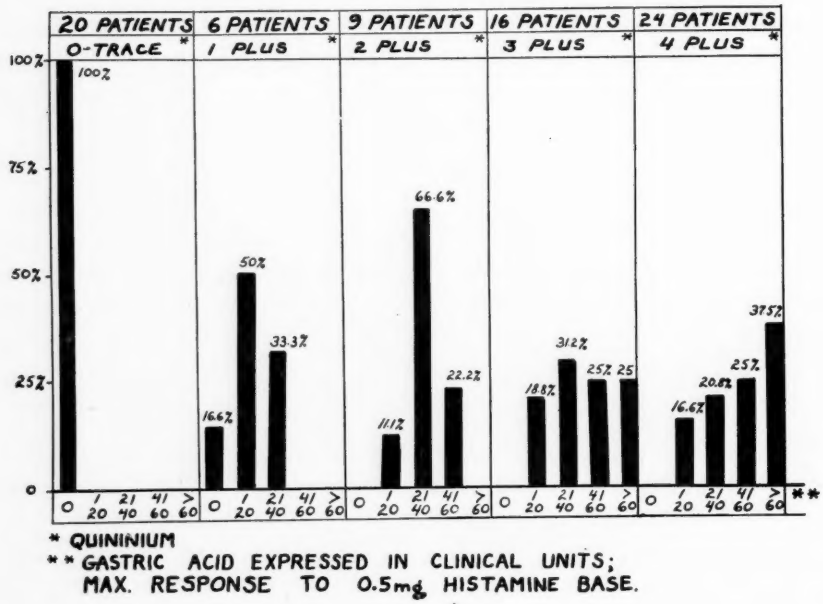


Fig. 2.

It had been our hope at the beginning of this investigation that the second hour urine specimen might give valuable evidence as to the degree of acidity present in the individual case. This hope was not justified as well be shown in our results. The quininium test is, however, reliable for the detection of

anacidity. Of 21 patients with histamine-proved anacidity, 20 had no detectable excretion of quinine in the urine. (Fig. 1.) One patient did have a one plus reaction in the second urine specimen. Unfortunately, we were unable to verify the absence of free hydrochloric acid in this patient by means of a second histamine—gastric tube test. The patient (A. T. No. 594,433) was a 59-year-old white woman without known gastrointestinal disease or other disease causing changes in the gastric acidity. She was used as a control patient and there is serious doubt that this patient had a persistent achlorhydria such as that found in pernicious anemia patients. To add more doubt as to the presence of persistent achlorhydria is the fact that all 15 patients with known persistent achlorhydria (pernicious anemia) showed consistently negative quininium tests. (Fig. 3.)

In those patients with free hydrochloric acid a comparison between the standard intubation—histamine test and the quininium test failed to reveal any consistent correlation or pattern. (Figs. 1 and 2.) In the 13 patients shown to have above 60 clinical units of free gastric acid (Fig. 1), 9 showed a four plus quininium reaction and 4 a three plus reaction. If, however, all patients showing a four plus reaction are analyzed (Fig. 2), it can be seen that only 9 of 24, or 37.5 per cent, had above 60 clinical units of free hydrochloric acid following histamine stimulation. The other 15 patients with a four plus quinine test showed free acid to be present in varying amounts from as low as 14 units to as high as 59 units. An examination of Fig. 2 further shows that patients with one, two, and three plus reactions had rather marked variation in the degree of hydrochloric acid concentration following histamine stimulation and gastric intubation. It is true that the tendency toward higher gastric acid concentration becomes more evident with higher quininium reactions. (Chart I.) It must be emphasized, however, that in the individual patient no definite conclusions can be drawn as to the concentration of acid present.

CHART I. AVERAGE GASTRIC ACIDITY CORRELATED WITH QUININIUM EXCRETION

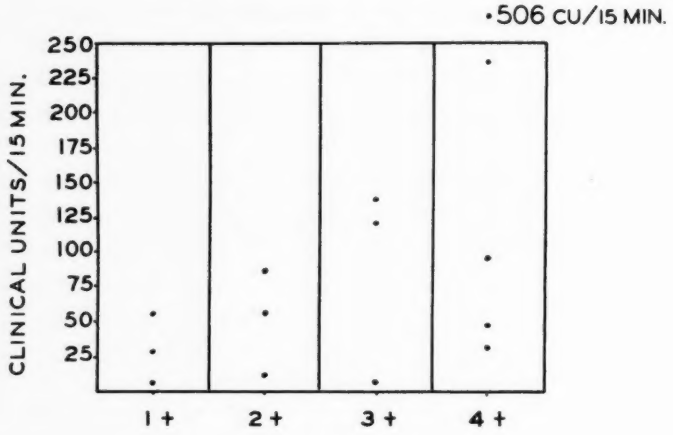
QUININIUM	TOTAL PATIENTS	AVERAGE ACIDITY*
4 plus	24	55.9
3 plus	16	45.0
2 plus	9	30.1
1 plus	6	15.8

*Clinical units free HCl after 0.5 mg. histamine base. Average of maximum responses.

We have compared the quininium test with the level of gastric acid concentration following histamine stimulation. We have done this since the latter test has come into such common usage and is so frequently employed to denote the presence of hypoacidity or hyperacidity. It is well known, however, that the rate of secretion or total amount of hydrochloric acid secreted per unit of time is a much more reliable and accurate indication of abnormal gastric secretory states. With this in mind we have compared the maximal 15 minute secretory rate as measured by intubation with the maximal excretion of quinine without intubation in 14 subjects. In these subjects caffeine stimulation (250 mg.

caffeine sodium benzoate orally) was utilized for both the tube and tubeless methods. Again the attempts at quantitative interpretation of the quininium test are unreliable in the individual patient although a trend toward higher secretory rates is again noted in the three and four plus reactors. (Chart II.)

CHART II. CORRELATION OF QUININIUM TEST WITH GASTRIC SECRETORY RATE FOLLOWING CAFFEINE STIMULATION



Since the presence of a one, two, three, or four plus reaction does not make this a quantitative test in the individual patient it would seem practicable to eliminate the analysis of the second hour urine specimen in all cases except those showing a negative or faint trace reaction in the first specimen. This would further simplify the test and still provide reliable evidence as to the absence or presence of free hydrochloric acid.

It has been repeatedly shown that duodenal ulcer patients tend to be hypersecretors and gastric ulcer patients normal or low secretors of gastric acid. Gastric carcinoma is usually associated with a low normal acidity or anacidity while pernicious anemia patients have a persistent achlorhydria. As can be seen in Fig. 3 duodenal ulcer patients showed a four plus reaction in 53 per cent of cases while no gastric ulcer patient showed a four plus reaction. One patient with a gastric ulcer had a negative quininium test. Gastric analysis in this patient using the Levin's tube and histamine stimulation likewise failed to reveal the presence of free hydrochloric acid. This patient was a 75-year-old white woman (S. S. No. 493,049) with senile depression and generalized arteriosclerosis. The ulcer failed to heal in spite of continued conservative therapy. Gastroscopic examination showed an atrophic mucosa with superficial erosions and one deep ulcer. It was the gastroscopist's opinion that this probably represented a malignant ulceration. Operation was not advised because of the patient's age and poor state of nutrition. The inclusion of this patient with gastric ulcer cases would, therefore, seem to be open to question. Patients with histologically proved gastric carcinoma showed either no free acid or a low titer quininium reaction. Pernicious anemia patients

were consistently negative when tested for free hydrochloric acid using either the quininium or standard gastric analysis tests. Those patients with hypertrophic gastritis showed normal to high quininium reactions. (Fig. 3.)

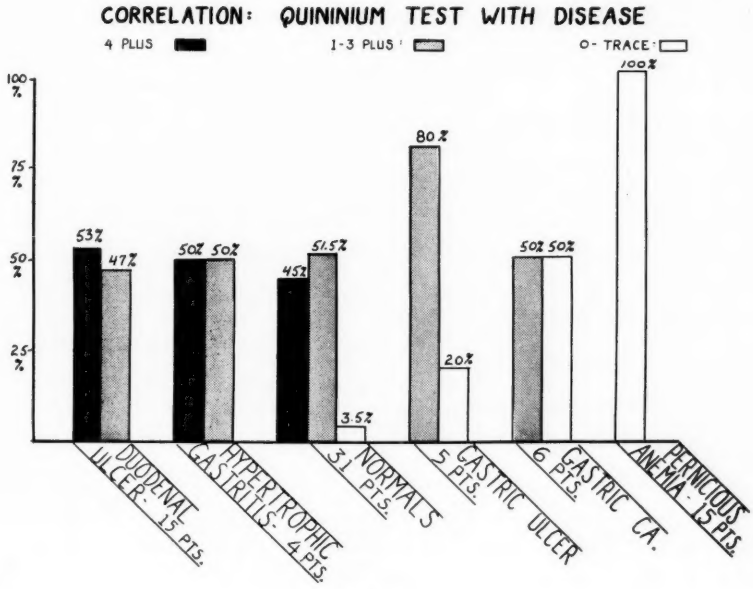


Fig. 3.

CONCLUSIONS

The quininium test is simple to do and does not require prolonged urine collections as is necessary with uropepsin determinations.[6]

The test has been extremely reliable in the detection of gastric achlorhydria.

The test is qualitatively reliable for the detection of free acid but the concentration or quantity of free acid present cannot be accurately predicted on the basis of the present grading system.

Urinary quininium determinations in 76 patients correlated well with the disease state present and compared favorably with the tube method of gastric analysis in the majority of cases.

This method should prove to be of value as a screening test for the detection of patients with achlorhydria.

The use of this test is indicated in those patients unable to tolerate the standard gastric tube either because of psychogenic or organic illness which precludes the use of a gastric tube. It is of extreme value in patients with partial gastrectomies of gastro-enterostomies because of the unreliability of the gastric tube method in these cases.

As with the uropepsin determination the use of this test as an aid in differential diagnosis of gastrointestinal lesions is limited. The presence of a

negative quininium test is strong evidence against the diagnosis of benign peptic ulcer. A one to four plus reaction is of little diagnostic value in the individual case.

The quininium test could be further simplified by the elimination of the second hour urine analysis in all cases except those showing a negative or faint trace reaction in the first hour specimen.

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STUDIES ON RENAL HYPERCHLOREMIC ACIDOSIS

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INTRODUCTION

THE disease of renal hyperchloremic acidosis is being recognized with increasing frequency as a cause of chronic acidosis, nephrolithiasis, nephrocalcinosis, osteomalacia or richets, and potassium deficiency.[1] It is only one of a number of specific renal tubular defects, such as renal glycosuria, the de Toni-Fanconi syndrome, pseudohypoparathyroidism, idiopathic hypercalciuria, and nephrogenic diabetes insipidus. Like most of these syndromes, renal hyperchloremic acidosis may sometimes be demonstrated to be a familial disorder.[2] Early diagnosis is of unusual importance because of the efficacy of prolonged replacement therapy with alkali, calcium, and vitamin D.[3] The basic feature of the disease is a failure of the normal renal mechanisms for the acidification of the urine. The remaining diverse features of the syndrome can be explained as resulting from this primary renal defect.[3] The studies which are reported here were undertaken to confirm and extend the previous observations on the altered physiology of this renal disorder. In particular, an attempt has been mad to evaluate the validity of some of the current theories about the nature of the specific tubular defect in renal hyperchloremic acidosis.

CASE HISTORIES

The case histories have been presented in detail in a previous report[2] and will be summarized only briefly here.

CASE 1.—The patient is a 28-year-old man, who was found to have bilateral nephrocalcinosis and pyelonephritis two years prior to the present study. There was no evidence of skeletal decalcification. His intake of milk had been greater than 1 quart daily for as long as he could remember. There was a normal oral glucose tolerance test without glycosuria. There was no increase of organic acids in the urine, to suggest the diagnosis of the de Toni-Fanconi syndrome. The characteristic pattern of hyperchloremic acidosis and a decreased ability to acidify the urine is described below. Two other male members of his family have been found to have renal hyperchloremic acidosis, and there is presumptive evidence for other cases in the family.

CASE 2.—The patient is a 20-year-old man, who was found to have nephrocalcinosis, nephrolithiasis, and azotemia (nonprotein nitrogen 82 mg. per cent) following the passage of several calculi one year prior to this study. There was no history to suggest previous renal disease. During the following year he had felt well except for occasional dysuria and recurrent renal colic. The nonprotein nitrogen returned to a normal value, but a low-grade pyelonephritis persisted. There was no evidence of skeletal decalcification. The patient had habitually drunk 1 to 2 quarts of milk daily. An oral glucose tolerance test was found to be normal with no glycosuria, and there was no increase of organic acids in the urine. The

Received for publication, Aug. 17, 1953.

characteristic findings in the plasma and urine are presented below. The family history did not suggest the occurrence of other cases. His only brother was found to have a normal serum chloride and carbon dioxide content, and no evidence of renal calcification.

METHODS

The patients were hospitalized at Walter Reed Army Hospital during the period of study. Most of the observations were carried out on a metabolic ward to insure accuracy of urine collections and control of the diet when necessary. Chemical determinations were carried out by the following methods: chloride,[4] serum calcium,[5] inorganic phosphorus,[6] alkaline phosphatase,[7] urinary ammonia,[8] urinary organic acids,[9] urinary titratable acidity,[10] and sodium and potassium.[11] "Arterialized venous blood" was drawn into oiled, heparinized syringes from the antecubital veins after the arm had been immersed in water at 43° to 46° C. for about ten minutes. Urine for acid-base analysis was collected under oil from an indwelling catheter or by direct voiding. The pH was immediately determined on whole blood and urine at room temperature, using a Beckman pH apparatus with an enclosed glass electrode. A correction factor of 0.014 pH unit per degree centigrade was subtracted to convert the pH of blood to that at 37° C. Urine pH was uncorrected for temperature. The carbon dioxide contents of blood and urine were determined manometrically.[12] The partial pressure of CO_2 (pCO_2) for blood was derived from the Singer-Hastings nomogram,[13] and for urine was calculated from the Henderson-Hasselbalch equation using a pK of 6.1 and an α CO_2 of 0.524. It is realized that some errors arise in the calculation of urinary pCO_2 by this method,[14] but these errors are not great enough to affect the interpretations of this paper. Urine for titratable acidity and ammonia was preserved with a small amount of toluene during the collection periods and kept under oil in the refrigerator until the following morning when the analyses were done.

Total exchangeable body sodium was measured by the dilution of Na^{24}. The isotope was administered intravenously as $Na_2{}^{24}CO_3$ by a single injection from a calibrated 10 ml. syringe. The solution was prepared to give an amount of isotope equal to 1.1 μC./kg. of body weight. The radiation from this amount does not exceed the accepted tolerance dose. Standards were prepared by a mock injection technique, and standard planchets were prepared to contain the same mass of solids as the unknown. An equilibration period of twenty-four hours followed the injection, and all urine was collected during this interval. Samples were prepared in duplicate and separate calculations were carried out on 4 cycles of 4,096 counts. After appropriate corrections for background and decay, total body sodium was determined by application of the formula:

$$\text{Exchangeable Na (meq.)} = \frac{\text{Counts injected} - \text{Counts excreted}}{\text{Counts/c.c. plasma} \div \text{meq. Na/c.c. plasma}}$$

RESULTS

1. *Acid-Base Regulation.*—Table I summarizes the hyperchloremic acidosis of the plasma and the inadequacy of the renal mechanisms for the preservation of base in the two patients. The titratable acidity and ammonia of the urine remain at low levels despite the challenge of a moderate, continued systemic acidosis. The pH of the urine in each case did not fall below 6.7 except during the experimental infusion of phosphate buffer as noted below. Included in the table for comparison are the urinary findings in a normal subject with a similar degree of acidosis secondary to the ingestion of ammonium chloride.[15] To emphasize this inability to excrete an acid load, 240 meq. of NH_4Cl were given to Case 2 over a 3 day period. The titratable acidity reached a maximum of 21.5 meq./24 hours and ammonia excretion rose to 40 meq./24 hours. The inadequacy of this renal response was reflected in a further fall of plasma pH

TABLE I. ACID-BASE PATTERN IN RENAL HYPERCHLOREMIC ACIDOSIS

	PLASMA				URINE		
PATIENT	CONTENT CO_2	pH	pCO_2	Cl–	pH	NH_3	TITRATABLE ACIDITY
	meq./L.		mm. Hg	meq./L.	6.7-7.5	meq./24 hr.	meq./24 hr.
Case 1	15.8-17.5	7.32-7.39	36-39	109-115	6.7-7.0	14.5-27.5	3.2-12.0
Case 2	13.9-20.2	7.25-7.31	36-40	114-121	c. 4.5-4.8	3.0-13.0	14.6-19.0
Acidotic control*	14.5					479	87

*A subject with a comparable degree of ammonium chloride acidosis is used as a control.[15]

from 7.31 to 7.23. Each patient exhibited the usual reversion of the plasma acid-base pattern to normal during continued therapy with 8.5 Gm. of sodium bicarbonate daily. These findings are confirmatory of those of other studies[3] and are necessary for the diagnosis of renal hyperchloremic acidosis.

2. *Sodium and Potassium.*—The serum levels of sodium were normal in both patients, as shown in Table II. Determination of the total body exchangeable sodium with Na^{24} was carried out twice in Case 1, with an intervening period of one month, and once in Case 2. It will be noted that there is excellent agreement on the repeated determination. The values for total exchangeable sodium were 32.3, 34.1, and 34.5 meq./kg., respectively. These figures are well below the average values, but fall just within the lowest range of normal found by Forbes[16] and Deane[17] for men.

TABLE II. TOTAL EXCHANGEABLE SODIUM AND PLASMA ELECTROLYTES IN PATIENTS WITH RENAL HYPERCHLOREMIC ACIDOSIS

	TOTAL EXCHANGEABLE SODIUM			AVERAGE PLASMA	
	PLASMA	TOTAL		Na	K
PATIENT	MEQ./L.	MEQ.	MEQ./KG.	MEQ./L.	MEQ./L.
Case 2	140	2070	32.3	139	3.5
Case 1	139	2408	34.1	139	4.1
Case 1	139	2430	34.5		
Normal	Deane[17]		34.1-50.7 av. = 40.9	138-145	4.0-4.8
	Forbes[16]		32.3-54.1 av. = 41.9		

Serum potassium was within the normal range in Case 1, but tended to be low in Case 2 (see Table II). Neither patient presented any evidence by history or physical examination of neuromuscular disability. An electrocardiogram in Case 2 did not show the changes which have been attributed to potassium depletion. To demonstrate the drain on cations secondary to failure of the cation conservation mechanism, Case 1 was placed on a carefully controlled daily intake of 1.3 Gm. of sodium, 2.6 Gm. of potassium, and 200 mg. of calcium for a period of one week. At the end of the week, the twenty-four-hour outputs of sodium, potassium, and calcium in the urine were determined. Urine sodium was 217 per cent, urine potassium 168 per cent, and urine calcium 58 per cent of that ingested.

3. *Calcium, Phosphorus, and Alkaline Phosphatase.*—The plasma values of calcium, phosphorus, and alkaline phosphatase were within normal limits on

repeated determinations in both patients. There was no evidence of decreased bone density. During studies several months apart Case 1 was found to have a urinary loss of 115 mg. and 283 mg. of calcium per twenty-four hours, after receiving a diet with less than 200 mg. of calcium for one week. An increase of calcium excretion has been emphasized by Albright and co-workers.[18] It is of interest that each patient had drunk at least one quart of milk daily for many years. This is the probable explanation of their freedom from osteomalacia and the presence of the marked degree of nephrocalcinosis.

4. *Water.*—Another of the well-localized functions of the distal renal tubule, in addition to its role in acid-base regulation, is that of the elaboration of a concentrated urine under the action of the antidiuretic hormone. In each patient there was a normal water diuresis, which was reduced following the intravenous injection of 400 milliunits of commercial Pitressin (Table III). The antidiuretic effect found in the patients, however, was not so marked as that in the controls. The ability to excrete a dilute urine was not impaired, but there was a maximal specific gravity of 1.010 in each case. Case 1 exhibited a moderate polyuria of about 3.5 liters daily on an ad lib. fluid intake, while Case 2 excreted a normal output of 1.9 liters while on the same control, neutral-ash diet. It is impossible to state whether this defective reabsorption of water is a primary tubular disorder, or whether it is secondary to the damage caused by extensive calcium deposition in the renal tissues and the attending pyelonephritis. At post-mortem examination the calcium deposits have been found to be most marked in the medulla and around the collecting tubules.[19]

TABLE III. IMPAIRMENT OF THE OLIGURIC RESPONSE TO ANTIDIURETIC HORMONE IN PATIENTS WITH RENAL HYPERCHLOREMIC ACIDOSIS

SUBJECT	WATER DIURESIS ML./MIN.	URINE FLOW AFTER PITRESSIN* ML./MIN.	AVERAGE 24 HOUR URINE VOLUME ML.
Case 1	17.0	3.8	3,450
Case 1	14.0	4.0	
Case 2	14.4	5.0	1,930
Control I	15.0	0.9	1,200 to
Control II	15.0	1.1	1,800

*The urine flows were measured 30 minutes after the intravenous injection of 400 milliunits of surgical Pitressin.

5. *Carbonic Anhydrase.*—It has been indirectly indicated by the use of inhibitors such as sulfanilamide, or 2-acetylamino 1,3,4-thiadiazole-5-sulfonamide (6,063) that the enzyme carbonic anhydrase is necessary for the normal acidification of the urine.[20, 21] From the use of these inhibitors there is also evidence that carbonic anhydrase is necessary for the secretion of hydrogen ions by the parietal cells of the gastric glands.[22] It seemed of interest to determine if there were a defect of this second hydrogen-ion-secreting mechanism, as an index of a possible widespread deficiency of carbonic anhydrase activity in patients with hyperchloremic acidosis. Case 1 was found to have a fasting level of 21 units of free acid with a total of 34 units. Case 2 had a fasting gastric juice pH of 3.0. There was, therefore, no gross deficiency in this mechanism in either patient. This does not rule out, however, a rate limitation in the secretion of acid by the gastric glands.

TABLE IV. EFFECTS OF THE INFUSION OF PHOSPHATE BUFFER

SUBJECT		PLASMA pH	PLASMA HCO_3 MEQ./L.	PLASMA pCO_2 MM. Hg	PLASMA P mM/L.	URINE FLOW C.C./MIN.	URINE pH	URINE HCO_3 MEQ./L.	URINE pCO_2 MM. Hg	URINE T.A. MEQ./MIN.	URINE NH_3 mM/MIN.	URINE P mM/MIN.
Case 1	control				0.78	9.0	7.20			.003		.013
(a)	buffer				4.60	11.0	6.60			.122		.320
Case 1	control	7.38	16.2	32	1.02	11.2	7.08	4.2	13	.013	.024	.019
(b)	buffer	7.34	19.1	40	5.95	8.0	6.42	3.0	30	.123	.033	.352
Case 2	control	7.25	15.7	40	0.83	13.7	6.71	3.8	25	.015	.040	.029
(a)	buffer	7.27	15.6	39	2.70	14.2	6.56	3.4	29	.065	.054	.180
Case 2	control	7.32	23.6	44	0.86	2.8	6.95	7.8	32	.006	.029	.016
(b)	buffer	7.41	22.1	34	2.70	4.7	6.81	5.2	28	.026	.034	.087
Latner & Burnard[23]	control	7.27	12.8	28	1.37	2.3	6.58	7.7	72	.018	.044	.027
Mean of 6 Cases	buffer	7.34	14.6	26	6.48	3.9	5.38	0.3	41	.272	.127	.306
Latner & Burnard[23] Mean of	control	7.20	14.4	34	1.10	2.5	4.72	0.04	34	.060	.103	.035
5 Acidotic controls	buffer	7.36	15.9	26	7.08	3.6	4.58	0.04	26	.518	.097	.585

Effect of the infusion of a phosphate buffer on the urine pH, bicarbonate, pCO_2, titratable acidity (T.A.), and ammonia. Recalculated data from Latner and Burnard are included for comparison. All of their figures are on the basis of 1.73 m^2 body surface.

THE RENAL TUBULE

THEORIES OF BICARBONATE REABSORPTION IN RENAL HYPERCHLOREMIC ACIDOSIS

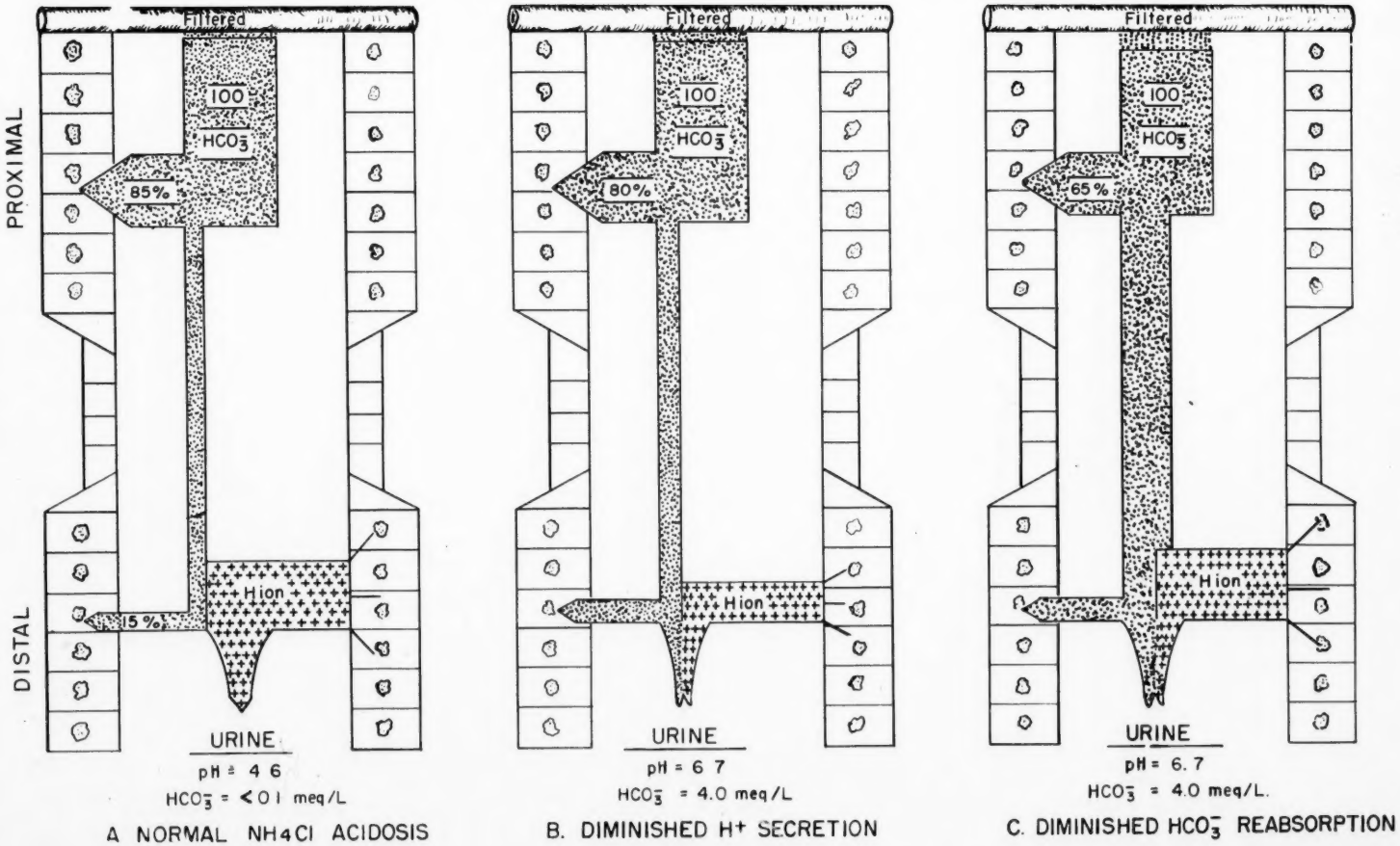

Fig. 1.

6. *Buffer Infusion.*—Latner and Burnard[23] studied a series of 6 infants and children with renal hyperchloremic acidosis and 5 controls made acidotic with ammonium chloride before and during the infusion of a phosphate buffer at pH 7.4. In the patients the buffer infusion caused a significant fall in urine pH, a fall of the previously elevated pCO_2, and a marked rise in titratable acidity and ammonia production. An isotonic Na_2HPO_4/NaH_2PO_4 buffer at pH 7.4 was infused intravenously at a constant rate for periods of sixty to one hundred twenty minutes on two occasions in both Case 1 and Case 2. The changes in urine pH, bicarbonate, pCO_2, titratable acidity, and ammonia were measured after equilibrium was attained. Table IV presents a summary of the changes observed, together with a summary of the results of Latner and Burnard. In confirmation of their work, a fall in the urine pH and a marked rise of the titratable acidity occurred during the buffer infusion. In general excretion levels as high as those in their series were not obtained, which may explain the finding of less pronounced changes in urine pH, titratable acidity, and ammonia. In contrast to their results, the initial pCO_2 in the urine was never found to be elevated. This anomalous rise in the urine titratable acidity during the infusion of a buffer at pH 7.4 has been previously described by Pitts for normal subjects, who stated, "It appears that conditions accepted as indicative of normalcy constitute a state of mild acidosis so far as the kidneys are concerned."[24]

7. *Bicarbonate Infusion.*—It has been postulated that the defective urinary acidification of renal hyperchloremic acidosis might be due to the flooding and neutralizing of a normally operating hydrogen ion exchange mechanism in the distal tubule by bicarbonate which had failed to be absorbed to the usual degree by the proximal tubule[23] (Fig. 1, *c*). This theory suggests a defective reabsorption of bicarbonate by the proximal tubules masking a normal secretion of hydrogen ions by the distal tubules. The absolute limit in the rate of H^+ secretion by the distal tubules has not been measured in man, although the limit in H^+ concentration in the urine seems to be about pH 4.4 to 4.5. A rate of .333 meq. of titratable acidity per minute has been measured in experimental acidosis in man during the infusion of a buffer.[26] It has been estimated that the true maximal rate of H^+ ion exchange must considerably exceed 0.5 meq./minute.[27] Thus in Patient 2 with a filtration rate of 2.2 meq. of bicarbonate per minute (125 ml./minute glom. filtration × .0175 meq./ml. bicarbonate in plasma) as shown in Table V, at least 25 to 30 per cent of the filtered bicarbonate would be required to neutralize such a normal, maximally operating H^+ ion secreting mechanism. The distal tubules are presumably operating maximally to acidify the urine, as the patient is in chronic acidosis. In the condition described by this theory, an elevation of the plasma bicarbonate would present a greater reabsorptive load to the defective proximal tubule, and failing to be absorbed normally, further flood and neutralize the distal acidifying mechanism. This should be reflected in a rise of urine bicarbonate and pH, perhaps to alkaline levels, although the systemic acidosis may not have been completely repaired. If an elevated pCO_2 in the urine represents the formation of carbonic acid by the interaction of H^+ and HCO_3^- in the distal tubule,[25] a rise of urine pCO_2

should be anticipated on elevation of plasma bicarbonate. Table V presents the changes in plasma and urine acid-base relationships during the intravenous infusion of a sodium bicarbonate solution in Case 2 sufficient to raise the plasma

TABLE V. ACID-BASE CHANGES FOLLOWING THE INFUSION OF BICARBONATE

CASE 2		PLASMA			URINE						
PERIODS		HCO_3 MEQ./L.	pH	pCO_2 MM. Hg	FLOW C.C./MIN.	HCO_3 MEQ./L.	pH	pCO_2 MM. Hg	HCO_3 MEQ./MIN.	% OF INC. LOAD EXC.	INULIN C.C./MIN.
Control	1	17.5	7.19	43	4.2	4.0	6.71	27	.017		128
60 minutes	2	17.7	7.19	44	2.8	4.1	6.73	26	.011		
	3	17.9	7.22	41	6.4	4.8	6.76	29	.031		
Bicarbonate	1	20.9	7.26	44	15.9	4.3	6.73	27	.068	12	124
Infusion	2	20.5	7.31	39	8.1	5.1	6.80	28	.041	5.7	
100 minutes	3	23.6	7.32	44	2.8	7.5	6.95	32	.021	1	
Recovery	1	22.5	7.39	37	5.9	5.7	6.82	30	.037	2.7	
50 minutes	2	22.1	7.41	34	4.7	5.2	6.81	28	.024	1	

A 5 per cent solution of sodium bicarbonate was infused intravenously at a rate sufficient to elevate the plasma bicarbonate conentration from 17.7 to 23.6 meq./L. over a period of 100 minutes.

bicarbonate from 17.7 meq./L. to 23.6 meq./L. over the course of 100 minutes. There was no significant change in the filtration rate of approximately 125ml./minute during the infusion. It will be noted that the pCO_2 did not rise significantly, and the urine pH rose only from 6.73 to 6.95. A variable, but quite small, percentage (mean = 4.5 per cent) of the increment in bicarbonate load due to the infusion appeared in the urine, the excretion tending to parallel the urine flow.

In summary, at least 25 to 30 per cent of the filtered bicarbonate would be required to neutralize a normal, maximally operating H^+ ion exchange mechanism when the patient's plasma bicarbonate was 17.5 meq./L. When the plasma bicarbonate was raised to 23.6 meq./L., however, there was no significant increase in urine bicarbonate (increase of 1 per cent) despite the increase in filtered load of 35 per cent. This failure to excrete a significant portion of the increment in filtered bicarbonate load, together with the resulting minimal rise in urine pH, is evidence against the occurrence of a defect in the proximal tubular absorption of bicarbonate as the only factor in renal hyperchloremic acidosis.

DISCUSSION

Renal hyperchloremic acidosis was demonstrated to be familial in one of the patients in this study, and nephrocalcinosis has also been noted in the sibling of another reported case.[28] The occurrence of most of the discovered cases in infants and children also suggests that the disease represents a congenital renal defect. Thorough investigation of the family should be made whenever this diagnosis is considered because of the importance of early and continued treatment with alkali to prevent progressive renal damage.

A complete physiologic description and localization of the tubular defect of renal hyperchloremic acidosis cannot be made until more information is available about the normal mechanisms for the absorption of bicarbonate and chloride and the exchange of H^+ ion for the Na^+ ion. Within the current con-

cepts of the kidney's role in acid-base balance, there are at least four mechanisms which singly, or in combination, could give rise to the characteristic chemical pattern in plasma and urine:

1. *A Primary Failure of the Distal Tubular Exchange of H^+ ion*[3] (Fig. 1, b).—Most of the features of renal hyperchloremic acidosis can be explained as occurring secondary to the simple failure of the distal acidifying mechanism. Urinary ammonia parallels changes in urine pH in a roughly linear relationship.[24] In general the ammonia excretions are not low for the pH's attained in renal hyperchloremic acidosis. The potassium deficiency which is frequently found in this condition could be caused by its excessive secretion by the distal tubule in lieu of H^+ ion.[21] When sufficient amounts of phosphate buffer are infused, however, the patients cannot only greatly increase the rate of H^+ ion secretion at the initial measured gradient (plasma pH to urine pH), but can also lower the urine pH significantly. Rates of acid secretion can be obtained which, if sustained, would be quite sufficient to repair the systemic acidosis and to prevent its recurrence. Latner and Burnard have cited this as indicative of a normal H^+ ion exchange mechanism, and believe that the infused phosphate in some manner increases the proximal reabsorption of bicarbonate.[23] This anomalous effect of a phosphate infusion has been noted for normal controls without acidosis[30] and is also found with infusions of sulfate, para-aminohippurate, and ferrocyanide.[29] The mechanism by which these substances increase urine acidity remains unclear.

2. *A Primary Failure of Bicarbonate Reabsorption in the Proximal Tubule*[23] (Fig. 1, c).—A corollary to this theory is that the unabsorbed bicarbonate floods and neutralizes a normally operating exchange of H^+ ion in the distal tubule. The failure of the bicarbonate infusion experiment to support this suggested pathogenesis of renal hyperchloremic acidosis has been discussed above. Latner and Burnard interpreted the elevated urinary pCO_2 values of their six patients as an index of an increased reaction of H^+ and HCO in the tubule to form carbonic acid. The two patients reported here were not found to have elevation of urine pCO_2. There is now considerable evidence that urine pCO_2 values different from those of extracellular fluid may result from the admixture of urine specimens with varying H^+ ion and buffer concentrations from the individual tubules,[14] and thus not represent a valid index of the interaction of H^+ and HCO_3^- in the distal tubule. The patients of Latner and Burnard were stated to be near normal in ammonia production, but they were compared with controls who had been acidotic for only eighteen hours. In view of the chronic acidosis of the patients and the marked increase in ammonia which occurs during the first forty-eight to seventy-two hours of acidosis, a valid comparison could be made only with controls who had been acidotic for at least this period of time. The anomalous fall of urine pH and increase of the urinary titratable acidity occurring when a phosphate buffer is infused into patients with renal hyperchloremic acidosis do not constitute evidence for this mechanism. It is as plausible to consider this an increased efficiency of H^+ ion secretion, as to consider it an "unmasking" of a normal H^+ ion tubular secretion by an increased

efficiency in the proximal reabsorption of bicarbonate. In summary, it seems unlikely that a primary failure to reabsorb bicarbonate in the proximal tubule represents the only renal defect in the patients whom we have studied.

3. *A Primary Increase in Chloride Reabsorption.*—To maintain the elevated extracellular chloride concentration of this disease demands an increased rate or degree of reabsorption of chloride from the glomerular filtrate or an increased intake of chloride. A reciprocal relationship between chloride and bicarbonate reabsorption by the renal tubule has been suggested,[31] although others have failed to confirm this finding.[32] If an abnormally increased chloride reabsorption were primary, the defective urinary acidification would result secondarily from the "bicarbonate flooding" mechanism discussed under *2* above, and presented in Fig. 1, *c*. The same evidence against the "bicarbonate flooding" mechanism is therefore equally valid against the primacy of an increased chloride reabsorption as the initiating derangement. It has been demonstrated that the acidosis of patients with renal hyperchloremic acidosis can be exacerbated by the administration of excessive amounts of NaCl.[3] This would hold true to a lesser extent for normal subjects also. It is interesting to note that hyperchloremic acidosis, occasionally associated with a deficiency of potassium and calcium, develops in a number of patients following bilateral ureterosigmoidostomy.[33] This has been attributed to the excessive reabsorption of chloride, probably as ammonium chloride, by the colon.

4. *A Combined Defect of Bicarbonate Reabsorption in the Proximal Tubule and of H^+ Exchange in the Distal Tubule.*—Normally about 80 to 85 per cent of the water in the glomerular filtrate is reabsorbed in the proximal convoluted tubule. Micropuncture studies have demonstrated minimal changes in pH within the proximal tubule.[34] Unless there are marked variations in pCO_2, therefore, an equal percentage of bicarbonate must normally be reabsorbed there, leaving only 15 to 20 per cent to be reabsorbed by the distal tubule. Berliner and co-workers[21] have found 30 to 40 per cent of the filtered bicarbonate in the urine of acidotic dogs under the influence of "6,063," a carbonic anhydrase inhibitor. This would seem to indicate that the reabsorption of bicarbonate by the proximal tubule, as well as the secretion of H^+ ion by the distal tubule, is dependent upon carbonic anhydrase activity, possibly representing the same fundamental process. The other possible explanations are: (a) a secretion or exchange mechanism for bicarbonate in the distal tubule, (b) gross errors in the estimates of the distribution of water reabsorption between the proximal and distal tubules, or (c) an action of "6,063" on some other unknown enzyme system. With the incomplete evidence at hand a defect of this common biochemical mechanism dependent on carbonic anhydrase, resulting in a simultaneous decrease in the proximal reabsorption of bicarbonate and in the distal exchange of H ion, represents a more plausible explanation of the impaired acidification of the urine in renal hyperchloremic acidosis than the previously presented hypotheses.

SUMMARY

A number of clinical and physiologic studies carried out on two men patients with renal hyperchloremic acidosis have been presented. The total

exchangeable sodium, measured with $Na,^{24}$ was found to be at the lowest range of normal in each patient. One patient had a low plasma potassium, but no clinical or electrocardiographic evidence of potassium deficiency. Four theories of the pathogenesis of renal hyperchloremic acidosis have been examined, and the evidence for and against each has been summarized. Since the reabsorption of bicarbonate and the secretion of the hydrogen ion probably represent the same fundamental process, it is suggested that a combined defect of these functions represents the best explanation for the impaired urinary acidification of this disease. An exact localization and description of the defect in the renal tubule must await a better understanding of the normal mechanisms for the controlled absorption of bicarbonate and chloride and the secretion of the H^+ ion by the kidney.

We are pleased to acknowledge the suggestion of Drs. Robert W. Clarke, Victor Sborov, Lawrence Kyle, and David Seligson. Miss Lois Liddle rendered valuable technical assistance.

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COMPARATIVE EFFECTS OF NEWER ANTICHOLINERGIC AGENTS ON HUMAN GASTRIC SECRETION

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INTRODUCTION

INHIBITION of acid gastric secretion occupies a prominent position in the present-day management of peptic ulcer. Atropine has been widely used for this purpose despite its well-known limitations. Recently newer agents have been synthesized with the hope of obtaining a more satisfactory drug of this kind. The present report concerns observations made on the comparative effects of eleven such drugs on human gastric acid secretion. This group includes atropine and 10 quaternary amines: Squibb preparations 2,963, 3,199, 3,505, 2,806, and 2,998, Banthine and Pro-Banthine (Searle), Antrenyl (Ciba), Prantal (Schering), and Ro2-3,773 (Hoffmann-LaRoche).

METHODS OF STUDY

The subjects consisted of 134 hospitalized patients, 25 of these serving as controls. Forty-three per cent of the test group and 48 per cent of controls had peptic ulcers, active or healed, while the remainder had a variety of diseases not related to the upper gastrointestinal tract.

With a few exceptions in the peptic ulcer group, none of the patients prior to study received any medications with a known effect on the gastrointestinal tract. Studies were done in the morning after an eight- to ten-hour fast and in most cases were completed on the same day. In a few instances it was necessary to test drugs on the day following control observations. The effect of each drug upon food-stimulated or basal gastric acid secretions was determined. Environmental factors were standardized for the entire group and maintained constant during control and test periods in each case. Bloody or bile-stained specimens were discarded and further contamination avoided by constant ejection of salivary secretions throughout each period of study. Specimens were titrated for free and total acid using Toepfer's reagent and phenolphthalein as indicators.

Food Stimulated Secretion Studies (Double Meal Technique).—A standardized meal of arrowroot biscuits and water was given, followed in fifteen to thirty minutes by intubation and collection of 5 to 10 ml. specimens every fifteen minutes for a control period ending two hours after the meal. The stomach was then aspirated completely, the tube withdrawn, and the test medication given. One hour later the same procedure of administration of the standard meal followed by intubation and collection of specimens was repeated. The effect of medication was observed by comparing the secretory response after each meal, the first serving as a control for the second. Results were graded as follows: (A) No effect. The average concentration of free hydrochloric acid in specimens obtained after the second meal

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This study was carried out with the aid of grants from the Charlton Fund of Tufts College Medical School, and E. R. Squibb & Sons.
Received for publication, Sept. 4, 1953.

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was less than 10 meq./liter lower than in those after the first meal. In 15 additional control subjects who received no medication but who were otherwise subjected to the same procedure as above, the average concentration of free hydrochloric acid in the second meal specimens was 5.3 meq./liter more than that of the first meal specimens (S.D. = ±7.4 meq./liter). The value of −10 meq./liter was greater than two times the standard deviation from the mean difference of +5.3 meq./liter in this group, and would include 95 per cent of variations occurring as the result of chance. In our patients the figure of −10 meq./liter corresponded to −20 per cent. (B) Definite effect. The average concentration of free hydrochloric acid in specimens following the second meal was more than 10 meq./liter (or 20 per cent) lower than those after the first. (C) Achlorhydria. This effect was recorded separately as representing maximum inhibition.

Basal Secretion Studies.—Patients with gastric hypersecretion with or without demonstrable peptic ulcer were the subjects of this study. A modification of the method of Polland and Bloomfield[1] was used. Intubation and complete aspiration of the stomach was carried out under basal conditions, followed by quantitative collection of ten-minute specimens using mechanical suction (Gomco) combined with continuous aspiration with a syringe by hand. After a satisfactory control period (6 to 9 specimens) the tube was clamped off and the test medication given. One hour later the stomach was reaspirated completely and collection of specimens continued for another two to three hours or longer. A similar procedure except for omission of medication was carried out in 9 patients who served as controls. In this group, however, specimens were collected continuously for three hours without interruption by clamping off the tube and a slow intravenous drip of normal saline (135 to 455 ml.) was run in during the period of study. In basal secretion studies results were recorded as the percentage of variation of free hydrochloric acid (calculated in total milliequivalents) between separate test and control periods of study. Achlorhydria when present was recorded separately as representing the maximum variation.

In both groups careful note was made of the presence or absence of "side effects" of medication such as xerostomia, mydriasis, blurring of vision, and bladder and bowel dysfunction.

RESULTS

Results with the eleven drugs studied in 110 patients have been tabulated in Tables I to IV. It will be seen that on the basis of potency they can be arbitrarily divided into the following four groups: (1) Atropine. (2) Antrenyl and Ro2-3,773. (3) Pro-Banthine, 2,963, Banthine and 3,199. (4) 2,998, 3,505, 2,806, and Prantal.

Atropine.—In order to obtain maximum effects for the comparative purposes of this study somewhat higher doses of atropine were used than those routinely employed in therapy. It was by far the most potent of the various drugs tested. (Table I.) Definite inhibition of secretion occurred in 6 of the 9 patients studied (including 3 with peptic ulcer) during double meal tests following both oral and parenteral administration of this drug. No cases of achlorhydria were seen. In one basal secretion test, however, a prolonged period of achlorhydria followed a similar oral dose. Dryness of the mouth was noted frequently in these cases including the majority of those showing an effect as well as in 2 of the 3 who did not.

From these results it appears that even in large doses atropine will be quite variable in its effect on gastric secretion. The ideal of complete inhibition will be seldom attained. Although an accurate evaluation of the relation between side effects and antisecretory effects at therapeutic levels would not be possible

from these studies, the variability of results obtained here with high doses, as well as common experience in therapy where smaller doses are the rule, would seem to indicate that side effects are unavoidable if amounts adequate to inhibit gastric secretion are used.

TABLE I. EFFECTS OF ATROPINE ON HUMAN GASTRIC SECRETION

DRUG AND NO. OF CASES	DOSE MG. (MG./KG.)	ROUTE		DOUBLE MEAL			BASAL SECRETION			SIDE EFFECTS	
		PO	IM	NO EFFECT	DEFINITE EFFECT	ACHLORHYDRIA	NO. CASES	% SUPPRESSION	ACHLORHYDRIA	XEROSTOMIA	CYCLOPLEGIA
Atropine 10 (4)*	1.28 (0.015-0.023)	7		2 (1)*	4 (1)*	-	1	-	1 (1)*	4	-
	1.28 (0.019-0.025)		2	1	1 (1)	-				2	1
	0.32 (0.005)		1	-	1	-				1	-
Total patients		7	3	3 (1)	6 (2)	-	1	-	1 (1)	7	1

*Parentheses indicate patients with peptic ulcer or hypersecretion.

Antrenyl and Ro2-3,773.—Antrenyl[2-4] (Oxyphenonium bromide:diethyl [2 hydroxyethyl] methylammonium bromide a-phenyl-cyclohexaneglycolate) and Ro2-3,773 (5) (1-methyl-3-benzyloyl-oxyquinuclidinium bromide) are both quaternary compounds which have been shown recently to be effective anticholinergic agents. Quantitatively they appear to be similar in their action. (Table II.) Observations on 19 patients will be seen to indicate an effective oral dosage range of 10 to 25 mg. for each. There was definite inhibition of gastric secretion with these amounts orally in four of eight patients receiving Antrenyl and in three of nine patients given Ro2-3,773. Achlorhydria occurred twice with the former and once with the latter drug. In an additional basal secretion study using Antrenyl, there was a 32 per cent reduction in secretion. The effectiveness of Ro2-3,773 appeared to be greater when given parenterally than when administered orally. In two patients given 5 and 10 mg. intramuscularly respectively, a pronounced inhibition of secretion occurred associated in each case with achlorhydria. In the latter case, a patient with an active obstructing duodenal ulcer, achlorhydria persisted for nearly seven hours until the test was terminated. Side effects, primarily dryness of the mouth, were noted in the majority of patients showing inhibition with either drug. Such effects were also noted in some cases in which no inhibition occurred.

Pro-Banthine, 2,963, Banthine, 3,199.—The pharmacology and clinical effectiveness of Banthine (Methantheline bromide: B-diethylaminoethylxanthene-9-carboxylate methobromide) have been reported in the literature for the past few years. Its isopropyl analogue, Pro-Banthine[6-10] (SC 3,171: B-diisopropylaminoethylxanthene-9-carboxylate methobromide) has been described recently as qualitatively similar in its action but more potent without any sig-

nificant increase in toxicity. Previous clinical studies in addition have indicated an effectiveness in inhibiting gastric secretion and motility with lessened side effects.

Our studies with Pro-Banthine failed to indicate any advantage over its analogue in respect to side effects. In addition, in double meal and basal secretion studies in which 30 to 60 mg. were given orally, considerable variability in

TABLE II. EFFECTS OF ANTRENYL AND Ro2-3,773 ON HUMAN GASTRIC SECRETION

DRUG AND NO. CASES	DOSE MG. (MG./KG.)	ROUTE		DOUBLE MEAL			BASAL SECRETION			SIDE EFFECTS	
		PO	IM	NO EFFECT	DEFINITE EFFECT	ACHLORHYDRIA	NO. CASES	% SUPPRESSION	ACHLORHYDRIA	XEROSTOMIA	CYCLOPLEGIA
Antrenyl 8 (1)*	10-15 (0.1-0.2)	7		3	1	2	1	(32)*	–	2	
	25 (0.3)	1		–	1	–				1	
Total patients		8		3	2	2	1	1 (1)		3	
Ro2-3,773 11 (4)	10 (0.1-0.2)	3		2	1	–				1 2 ?	
	5-10 (0.1-0.2)		2	–	–	1 (1)	1	–	1 (1)	2	
	20-25 (0.3-0.4)	3		2 (1)	–	1 (1)					
	30-35 (0.5-0.6)	3		2	1	–				3	
Total patients		9	2	6 (1)	2	2 (2)	1		1 (1)	6 2 ?	

*Parentheses indicate patients with peptic ulcer or hypersecretion.

effect on gastric secretion was noted (Table III.) Only 3 of the 8 patients in this group showed a definite inhibition of secretion with achlorhydria occurring in two. In spite of this, side effects, chiefly xerostomia, were noted frequently, including three of those in whom inhibition did not occur. By contrast Banthine orally in 100 to 200 mg. doses produced inhibition in all but 1 of 9 cases studied (Table III.) Side effects were also present in the majority of these patients.

2,963[11] (dimethylethyl 3-[n-propyl] benzilate ammonium bromide) and 3,199[11-12] (N-diethylaminoethyl-N-methyl-benzilamide methobromide) have been shown experimentally in animals to have high atropine-like activity, the former being about twice as potent as the latter in this respect. 3,199 in addition is one of the few quaternary compounds with an amide linkage reported to have this kind of action. The 36 patients studied with these drugs tended to confirm this impression as to their relative potency. (Table III.) Orally both were found effective in inhibiting gastric secretion, 3,199 being only slightly less potent than Banthine in this respect and 2,963 slightly more so. Definite inhibition was demonstrated on double meal studies in about two-thirds of those receiving 3,199 in doses of 75 to 250 mg. orally and in nearly two-thirds of those given 2,963 in doses of 25 to 75 mg. orally. In basal secretion tests similar results were observed. Achlorhydria occurred five times with the use of the former drug and seven times with the latter. While little difference in results could be seen with 3,199 in doses of 150 to

250 mg. as compared to 75 to 100 mg., with 2,963 increasing the dosage to 100 to 200 mg. resulted in a slight increase in the number of positive results. Side effects, chiefly dryness of the mouth, were observed in the majority of patients studied with both drugs.

TABLE III. EFFECTS OF PRO-BANTHINE, 2,963, BANTHINE AND 3,199 ON HUMAN GASTRIC SECRETION

DRUG AND NO. CASES	DOSE MG. (MG./KG.)	ROUTE		DOUBLE MEAL			BASAL SECRETION			SIDE EFFECTS	
		PO	IM	NO EFFECT	DEFINITE EFFECT	ACHLORHYDRIA	NO. CASES	% SUPPRESSION	ACHLORHYDRIA	XEROSTOMIA	CYCLOPLEGIA
Pro-Banthine 8 (1)*	30 (0.5-0.7)	2		1	1	–				1 1 ?	
	45 (0.5-0.9)	5		3	–	1	1	–	1 (1)*	4	1 ?
	60 (0.9)	1		1	–	–				1	
Total patients		8		5	1	1	1		1 (1)	6, 1 ?	1 ?
2,963 19 (9)	25-75 (0.4-1.3)	10		3 (1)	3 (1)	1	2	25	1 (1)	7 2 ?	3
	100-150 (1.5-2.2)	8		1 (1)	2	2 (1)	4	(81)	3 (3)	6	1
	200 (2.0)	1		–	1	–				1	–
Total patients		19		4 (2)	6 (1)	3 (1)	6	2 (1)	4 (4)	14, 2 ?	4
Banthine 9 (8)	100 (1.5-2.1)	2		–	1 (1)	–	1	(83)	–	1	–
	150 (1.9-3.0)	5		–	2 (2)	2 (2)	1	(0)	–	4 1 ?	2
	200 (2.4-2.6)	2		1	1 (1)	–				1	–
Total patients		9		1	4 (4)	2 (2)	2	2 (2)		6, 1 ?	2
3,199 17 (12)	25 (0.3)	1		1 (1)	–	–				–	–
	35-50 (0.4-0.6)		2	–	–	–	2	–	2 (1)	2	2
	75-100 (0.9-2.6)	7		2	2 (1)	1 (1)	2	(96)	1 (1)	3 1 ?	–
	150-200 (2.1-3.1)	5		1 (1)	1	–	3	(85) (70)	1 (1)	5	2
	250 (3.1-3.8)	2		–	2 (2)	–				2	–
Total patients		15	2	4 (2)	5 (3)	1 (1)	7	3 (3)	4 (3)	12, 1 ?	4

*Parentheses indicate patients with peptic ulcer or hypersecretion.

Prantal, 2,998, 3,505, 2,806.—The four drugs in this group were the least effective of those tested. (Table IV.) Prantal[13, 14] (N, N dimethyl 4-piperidylidene 1, 1 diphenylmethane methyl sulfate) was reported originally to have a more or less selective action on the gastric parasympathetic ganglia, causing fewer side effects than other anticholinergic agents.

In our studies with this drug a variable effect on gastric secretion was obtained with oral doses of less than 350 mg. In this range only one patient out of 6 showed a definite inhibition of secretion; this result was associated with achlorhydria. Above 400 mg. orally a more consistent effect occurred, 4 of 5 patients in this group showing an inhibition without achlorhydria.

Dryness of the mouth was observed frequently but was inconsistent, occurring nearly as often in those in whom inhibition was demonstrated as in those in whom it was not. The effectiveness of the parenteral form of this medication was not studied.

2,998[11, 15] (2-hydroxyethyl methylpiperidinium bromide, 1 benzoylcyclopropanecarboxylate), 3,505[11, 15] (diisopropyl [?-hydroxyethyl] methylammonium bromide of 1-benzoylcyclopropane carboxylate), and 2,806[11, 15] (2-ethyl [1-benzoyl cyclopropane-1-carboxylate] methyl diethyl ammonium bromide) are members of a group of benzoyl cyclocarboxylic acid esters of diethylaminoethanol reported to have anticholinergic activity in animals. These three drugs were found to be ineffective orally in doses up to 350 mg., 300 mg., and 750 mg., respectively, in 14 patients tested with double meals. Side effects were noted only at the upper limits of dosage with the first two. 2,806 was also given parenterally in doses up to 220 mg. By this route it was effective in inhibiting secretion in 4 patients, one of whom developed achlorhydria in a basal secretion study. Side effects occurred in all of this group.

TABLE IV. EFFECTS OF 2,998, 3,505, 2,806, AND PRANTAL ON HUMAN GASTRIC SECRETION

DRUG AND NO. CASES	DOSE MG. (MG./KG.)	ROUTE		DOUBLE MEAL			BASAL SECRETION			SIDE EFFECTS	
		PO	IM	NO EFFECT	DEFINITE EFFECT	ACHLORHYDRIA	NO. CASES	% SUPPRESSION	ACHLORHYDRIA	XEROSTOMIA	CYCLOPLEGIA
2,998	100-350	6		6	–	–				1	–
6	(1.6-4.3)									1 ?	
Total patients		6		6						1	
										1 ?	
3,505	200-300	2		2	–	–				2 ?	–
3	(2.6-4.7)										
	500	1		–	1	–				1	
	(5.8)										
Total patients		3		2	1					1	
										2 ?	
2,806	150-600	4		4 (3)*	–	–				–	–
9 (6)*	(2.0-8.9)										
	750	1		1 (1)	–	–				–	–
	(15.6)										
	100-220	–	4	–	3 (2)	–	1		1	4	2
	(1.7-3.1)										
Total patients		5	4	5 (4)	3 (2)		1		1	4	2
Prantal	200-350	6		4 (1)	–	1	1	(0)	–	4	–
11 (5)	(2.8-4.7)										
	400-700	5		1 (1)	4 (2)	–				1	–
	(5.1-9.8)									2 ?	
Total patients		11		5 (2)	4 (2)	1	1	1 (1)		5	
										2 ?	

*Parentheses indicate patients with peptic ulcer or hypersecretion.

Comment.—In control studies under the conditions described the basal gastric secretion was found to be more variable than the response to food stimulation (double meal test). Variability in control basal secretion studies was also noted originally by Polland and Bloomfield,[1] but has since been largely ignored by others using the test to evaluate antisecretory agents. In basal

secretion tests with the various drugs, frequently it was difficult to determine the presence or degree of anticholinergic effect except when marked inhibition or achlorhydria was produced. Hence, in general the double meal technique was felt to be preferable. Since in the light of present theories of gastric function it appears likely that the two techniques measure different phases of gastric secretion, information about both should be useful in complete evaluation of a drug. Inhibition of resting as well as food-stimulated gastric secretion represents an important therapeutic objective in the management of peptic ulcer. Too, duration of drug effect is more readily estimated by means of the basal secretion test.

With the eleven anticholinergic agents tested the ideal of achlorhydria was seldom attained in double meal studies. As anticipated it occurred more often during the study of basal secretions. With all drugs considerable variability of results was observed with similar doses in different patients. The effect obtained was not always proportional to the dose used; high doses did not uniformly result in greater inhibition than smaller ones. In evaluating results in double meal tests 20 per cent suppression of secretion was found to indicate a definite inhibition in our patients. It will be readily appreciated that in cases of hypersecretion this is not a therapeutic triumph. Yet, with the usual doses none of the drugs studied could be shown to do consistently better than this. Most drugs tested were found to be more potent when administered parenterally than when given orally. It would seem that variations in absorption and transport by the oral route might be important in this respect.

Despite claims to the contrary none of the drugs studied in this group appeared to have any clear or definite superiority over the others from the standpoint of lessened side effects. With few exceptions such side effects, chiefly xerostomia, were seen whenever inhibition of secretion occurred. In some cases they were also observed in the absence of inhibition of acid production. It would seem that for the present such undesirable side effects must be accepted as a necessary condition for effective anticholinergic therapy.

It should be emphasized that these tests were conducted with single doses of the various drugs and no conclusions concerning duration of action, tolerance, or cumulative effects would be possible from them. Duration of action would be an important therapeutic consideration in itself and because of the possibility of a rebound phenomenon. It is also unknown whether these drugs will continue to produce the same effects with constant use over a period of months. There is, however, suggestive clinical evidence of development of tolerance in some patients who report less dryness of the mouth after continued administration of the same dose. The possibility of cumulative effects should also be taken into account as they would be of obvious advantage in therapy. In a condition like peptic ulcer requiring prolonged administration of medication these points demand clarification by future studies.

SUMMARY

The effects of single oral or parenteral doses of 11 anticholinergic drugs on human gastric secretion are reported. Considerable variation in response to

similar doses of the same drug was observed from patient to patient. Achlorhydria was seen uncommonly after oral administration of large doses of the more potent agents studied. Smaller parenteral doses of most agents, however, produced achlorhydria more frequently, suggesting inequalities in absorption, transport or metabolism of orally administered medication. Correlation between gastric antisecretory effects and the production of side effects such as xerostomia was striking in that no drug consistently inhibited secretion without these "side effects." No drug appeared to have a clear superiority over the others in relation to lessened side effects when used in doses adequate to inhibit gastric acid secretion. The following approximate single oral doses of the drugs studied will reduce fasting gastric secretion in patients with ulcer or hypersecretion, or food-stimulated secretion in normals by 20 per cent or more: Atropine, 1.3 mg.; Antrenyl, 10 to 25 mg.; Ro2-3,773, 10 to 35 mg.; Pro-Banthine, 30 to 60 mg.; 2,963 (Squibb), 75 to 125 mg.; Banthine and 3,199 (Squibb), 100 to 150 mg.; Prantal, 3,505, 2,998, and 2,806 (Squibb), 400 to 700 mg.

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STUDIES ON PATHOGENESIS OF AMYLOIDOSIS

II. Effect of Nitrogen Mustard in Inducing Amyloidosis

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AMYLOIDOSIS has been produced experimentally by various means, including repeated parenteral injections of bacteria, antigens, casein, nucleotides, human serum, sulfur, etc.

The subcutaneous injection of nutose (sodium caseinate) into mice, a method introduced by Kuczynski, 1923,[1] has been shown to be a most effective way of producing amyloidosis.

Experiments already published[2] showed effects of cortisone and ACTH in inducing (or promoting) amyloidosis. In mice, treated for several weeks with injections of casein, insufficient to cause amyloid deposition, injections of cortisone for four to five days promptly resulted in the appearance of amyloid in the spleen.

Amyloid formation in the spleen and other organs was found to be preceded by a marked proliferation of cells showing pyronine-positive material in cytoplasm, such as plasma cells. The suppression of pyroninophilic cells in experimental conditions induced by cortisone was considered a basic alteration during the period of amyloid formation.

Since the principal systemic effect of the mustard compounds (a degeneration of the hemopoietic organs leading to involution of the spleen, lymphatic tissue, and thymus) in many respects resembles the depressive effects of cortisone, the possibility existed that nitrogen mustard, like cortisone, under certain conditions promotes amyloid formation.

The objective of the present study was to see whether or not nitrogen mustard actually promotes amyloid deposition in mice previously treated with injections of sodium caseinate for several weeks.

A case of Hodgkin's disease recently observed in which amyloidosis followed treatment, *inter alia,* with nitrogen mustard (Erasol) is included in the paper.

MATERIAL AND METHODS

Twenty-one female mice of a C_3H-strain, weighing 15 to 25 grams (Table I) and fed on an oatmeal diet ad lib. during the experiment and for the preceding eight days, were given daily subcutaneous injections of 0.5 ml. of a 5 per cent casein solution in 0.25 per cent NaOH. In the course of thirty days 28 injections in all had been given, after which the treatment with casein was discontinued in all the animals. The next day a laparotomy with ligation of the distal pole of the spleen for histologic examination was then done

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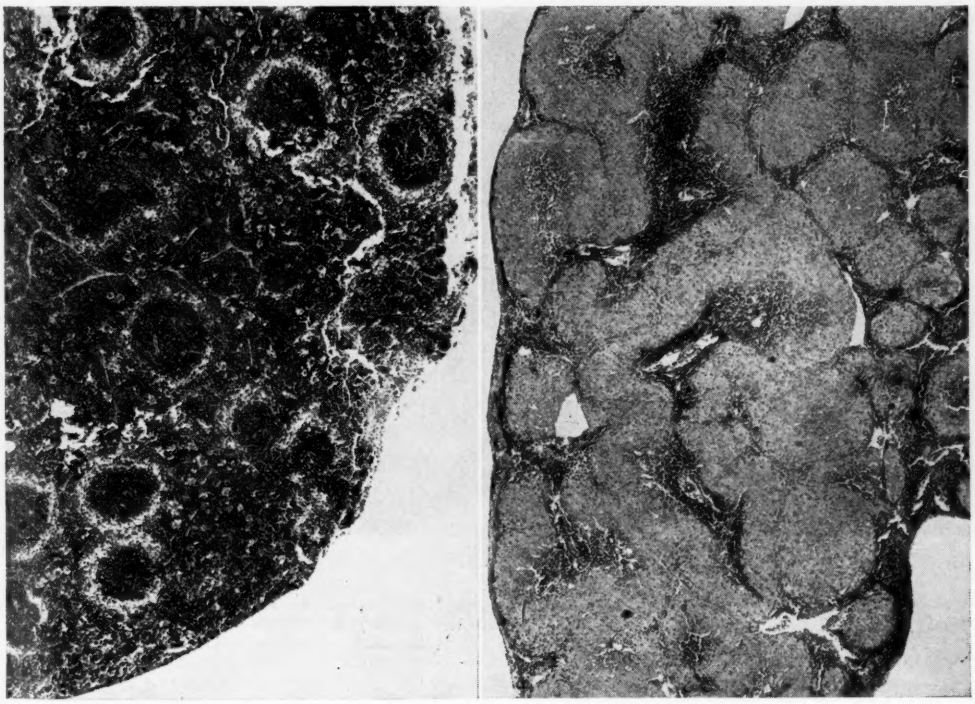
Aided by grants from the Danish League against Rheumatism and King Christian X's Foundation.
Received for publication, Aug. 27, 1953.

TABLE I. EFFECT OF NITROGEN MUSTARD IN PRODUCING AMYLOIDOSIS IN MICE PREVIOUSLY TREATED WITH INJECTIONS OF SODIUM CASEINATE

GROUP	MICE NO.	CASEIN 3/9 4/13	BIOPSY OF SPLEEN 4/14	NITROGEN MUSTARD DOSE MG./KG. 4/16	4/18	4/20	BODY WEIGHT 3/9	4/16	4/22		WEIGHT OF SPLEEN (MG.) IN NONOPERATED	POST-MORTEM FINDINGS OF AMYLOID SPLEEN
I	22		Mild degree of amyloid deposition in perifollicular zone cf. Figs. 1, *A* and 2, *A*				22.5	24.0	24.0	All animals of the four groups killed 4/22		Mild to moderate cf. Fig. 3, *A* and *B*
	24						21.0	24.0	24.0			
	25	+			0		19.5	22.0	21.5			
	26						15.0	17.0	18.0			
	47						18.5	21.0	21.0			
II	23			2.5	5	10	20.5	23.5	16.0			Maximal cf. Figs. 1, *B* and 2, *B*
	27			2.5	2.5	5	18.0	23.0	16.5			
	29			2.5	2.5	5	18.0	20.0	16.0			
	33	+		2.5	2.5	5	25.0	24.0	20.0			
	35			2.5	2.5	5	21.0	22.0	17.0			
	46			2.5	2.5	5	17.0	19.0	14.0			
	48			2.5	2.5	5	15.0	18.0	14.5			
III	36		No operation				18.5	23.0	23.0		125	Mild
	37	+			0		18.0	21.0	20.0		92	
	38						19.5	24.0	23.0		205	
	39						19.0	23.5	22.0		118	
IV	40		No operation	2.5	5	10	18.0	21.5	+21.4		85 (4/21)	Maximal
	41			2.5	2.5	5	18.0	24.0	17.5		77	
	43	+		2.5	2.5	5	20.0	25.0	20.0		65	
	44			2.5	2.5	5	17.5	22.0	15.0		82	
	45			2.5	2.5	5	15.5	19.0	15.0		47	

under ether anesthesia in 12 of the animals (Groups I and II). The other 9 animals (Groups III and IV) were not operated on.

Seven of the mice operated on (Group II) and 5 of those not operated on (Group IV) were then given 3 subcutaneous injections of methyl-di-(2-chloro-ethyl)-amine hydrochloride (HN_2·HCl) (''Erasol'' IDO) at intervals of two days. In most of the animals, 2 injections were given equivalent to 2.5 mg. per kilogram body weight and 1 injection equivalent to 5 mg. per kilogram, while the two last doses were doubled (5 and 10 mg./kg.) in two of the subjects (Table I). The dose was calculated in proportion to a weight of 20 grams, regardless of the slight variations in the weight of the animals (15 to 25 grams), and was given in the dilution 1:100 with a sterile NaCl solution, so that 0.5 ml. of the dilution corresponded to 2.5 mg./kg. for a mouse weighing 20 grams. Two days after the last injection the animals of all groups were weighed and then killed with ether. However, 1 of the animals (40) died the day after the last injection. Pieces of various organs were fixed in 4 per cent formalin and examined histologically after staining with hematoxylin and eosin, by the van Gieson-Hansen and the Unna-Pappenheim methods and with methyl violet and Congo red stain for amyloid. In cases in which biopsy of the spleen had not been performed (Groups III and IV) this organ had been weighed.



A. *B.*

Fig. 1.—*A*, Mouse 29. Biopsy of spleen before treatment with nitrogen mustard. Mild degree of amyloid deposition in the perifollicular zone. (Hematoxylin and eosin stain. ×40; reduced ⅙.)

B, Diffuse amyloidosis in spleen of same animal as in *A*, after further administration of nitrogen mustard. (Hematoxylin and eosin. ×40; reduced ⅙.)

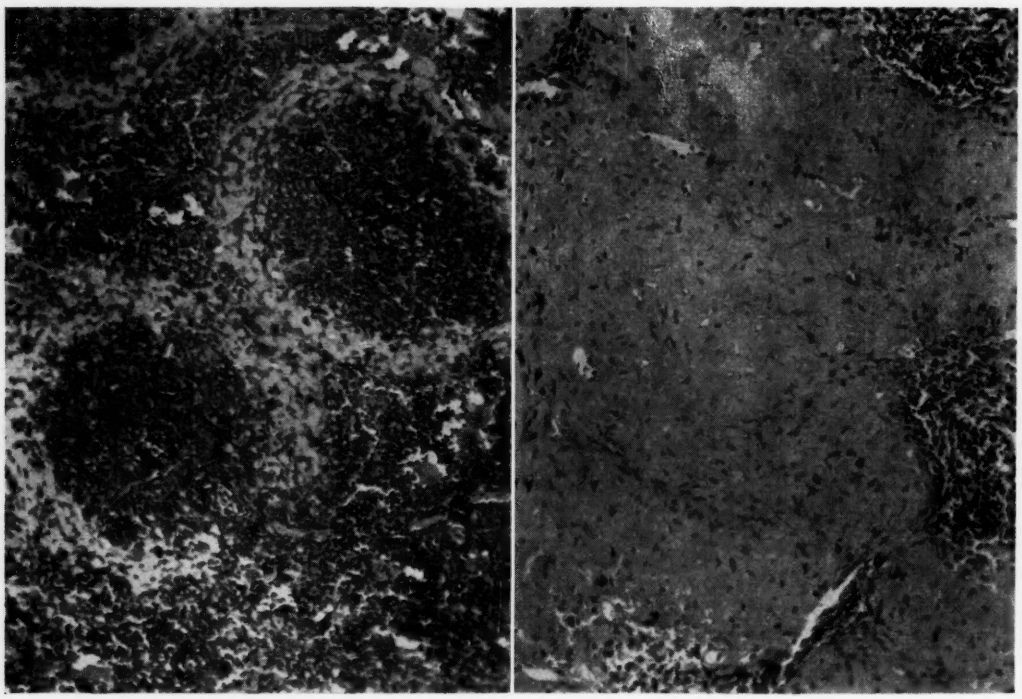
RESULTS

The experimental data are listed in Table I.

Histologically the biopsies of the spleen (animals of Groups I and II) removed at the time between discontinuance of the casein injections and the

administration of nitrogen mustard showed only a very early stage of amyloid deposition in the perifollicular zone (Figs. 1, *A* and 2, *A*) with rather narrow rings of homogeneous precipitates. As in our previous experiments in casein-treated mice, the Unna-Pappenheim stain revealed a very marked accumulation of pyroninophilic cells, including plasma cells and other reticuloendothelial cells, indicative of a stage of active proliferation, which was considered a typical precursor stage in the pathogenesis of amyloidosis.[2]

The animals of Groups II and IV which had been treated with nitrogen mustard showed a pronounced loss of weight in comparison with the controls which had been treated only with casein (Groups I and III).



A. *B.*

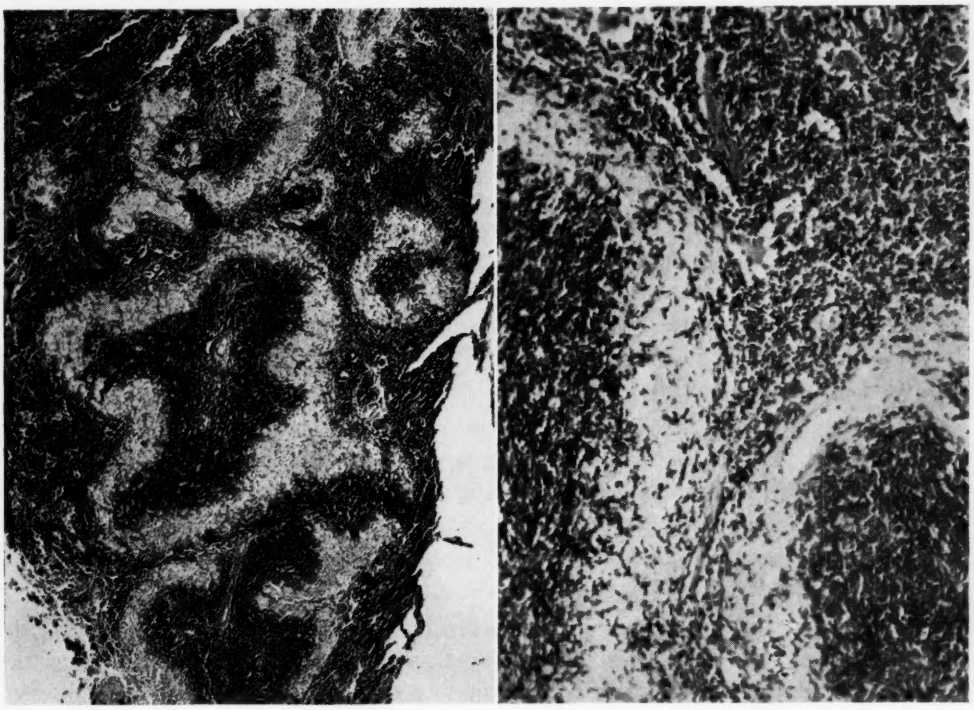
Fig. 2.—*A*, Higher magnification of the same section as in Fig. 1, *A*. Spleen before treatment with nitrogen mustard. (Hematoxylin and eosin. ×120; reduced ⅙.)

B, Higher magnification of section in Fig. 1, *B*, showing diffuse amyloidosis after administration of nitrogen mustard. (Hematoxylin and eosin stain. ×120; reduced ⅙.)

In the animals that had been treated but not operated on (Group IV) the spleen was much reduced in size, weighing 47 to 85 mg. (average 71 mg.). In the controls it weighed 92 to 205 mg. (average 135 mg.)

The spleen in the animals treated with casein injections only (Groups I and III) compared with the biopsy findings showed a more or less moderate increase in amyloid deposition with somewhat broader rings of homogeneous substance in the perifollicular zone (Fig. 3, *A* and *B*)—somewhat more pronounced in animals operated on than in those not operated on—whereas the histologic picture of the spleen was completely changed in all the 12 animals

(Groups II and IV) treated with nitrogen mustard. The organ consisted of large, massive amyloid depositions, almost confluent and separated only by very sparse accumulations of lymphocytes and, in a few cases, pyroninophilic cells. In all 12 cases the amyloidosis in the spleen was almost diffuse (Figs. 1, *B* and 2, *B*) and of a much severer degree than in the corresponding controls. The deposits gave a positive reaction with Congo red, but only a few cases reacted with methyl violet stain. In addition, focal amyloid formation in other organs (kidney, liver, adrenal, and intestine) was more frequent in the animals treated with nitrogen mustard (whether operated on or not), whereas only



A. *B.*

Fig. 3.—*A*, Spleen of control animal (mouse 25) treated with casein injections, but not with nitrogen mustard. (Hematoxylin and eosin stain. ×40; reduced ⅙.)

B, Higher magnification of the same section as in *A*. (Hematoxylin and eosin stain. ×120; reduced ⅙.)

the spleen was involved in the 4 controls treated with casein but not operated on (Group III). In these experiments, the laparotomy with biopsy of the spleen apparently caused a moderate increase of the amyloid deposition, whereas the treatment with nitrogen mustard induced an excessive degree of amyloid formation in all the animals.

DISCUSSION

The chloroethyl amines are known to be toxic to cells which are in a state of active proliferation. The chromosome fragmentation which is induced is

characteristic to the mustard, and because of the similarity of effects with those produced by x-rays these compounds have been termed "radiomimetic."

The effect of nitrogen mustard in inducing (or promoting) amyloidosis adds weight to the conception[2] that a suppression of mesenchymal cells which are in a state of active proliferation is involved in the basic mechanism of the formation of amyloid.

Previously it has been shown that the administration of cortisone results in a wide-spread decrease in number of plasma cells in the spleen and proliferating pyroninophilic cells of the glomeruli in experimental glomerulonephritis.[2, 3, 4] In mice treated with injections of sodium caseinate administration of cortisone would constantly promote the amyloid formation.[2]

It was proposed that experimental amyloidosis induced by various methods is dependent on the same basic mechanism, as it is well established that a variety of stimuli produce a proliferation of pyronine-positive cells in the spleen and other organs. The active stage of cellular proliferation was found to be maintained by ascorbic acid.[2]

Recent studies[5] revealed a marked *initial* stage of proliferation of pyronine-positive cells in the spleen of scorbutic guinea pigs, followed by a stage of regression in the chronic stage. The fact that the common organic changes and the electrophoretic pattern of the serum proteins closely reflect the findings during amyloid formation in general[5] may clarify the pathogenesis of amyloidosis in chronic scurvy observed by Pirani and co-workers, 1949.[6]

As the systemic intoxication produced by the mustard compounds in rats resembles a pattern of organic changes of Selye's alarm reaction[7] in many respects, this has been considered in interpreting some toxic effects of the drug (Karnofsky and associates.[8]) These authors stated that the damage to the bone marrow is rapidly completed and presumably a direct action of the agent, and not indirectly due to the alarm reaction.

Since cortisone and nitrogen mustard in experiments promoted amyloid deposition by an inhibition of proliferating mesenchymal cells, it might be expected that the therapeutic administration of these compounds was followed in certain cases by amyloid deposition. In a case of rheumatoid arthritis of only three years duration West and Newns[9] observed an enormous amyloidosis of the liver following treatment with cortisone for several months. Six months before the treatment a liver biopsy showed no signs of amyloid.

Amyloidosis in Hodgkin's Disease Treated With Nitrogen Mustard.—A 24-year-old man, admitted to Department A of the University Hospital, Copenhagen (Case record 2,795/52), gave a history of enlargement of the superficial lymph nodes, fatigue, and slight fever for a period of five months.

The *physical examination* revealed enlargement of the axillary lymph nodes. There were no palpably enlarged viscera in the abdomen. A roentgenogram of the chest taken on admission revealed enlarged lymph nodes of the mediastinum.

Biopsy of lymph nodes showed Hodgkin's disease.

Laboratory findings (on admission). *Blood:* Hemoglobin 78 per cent, erythrocytes 4,290,000 per cubic millimeter, leukocytes 10,000 per cubic millimeter. Sedimentation rate (Westergren) was 108 mm. in 1 hour. *Urine:* Albumin, negative. On Dec. 13, 1951,

a course of 12 consecutive intravenous injections of Erasol (nitrogen mustard) (total of 38 mg.) was begun. The first three injections were of 4 mg., the others of 3 mg. In addition a series of x-ray therapy was directed to the chest, neck, axillae, and groin (Dec. 18, 1951 to Feb. 6, 1952). From 400 to 1150 r were delivered to ten portals.

Following treatment the patient had a complete remission. The temperature fell to normal, the neoplastic lesions regressed and sedimentation rate decreased to 11 mm. (Feb. 1, 1952). Eight months later he was readmitted with fatigue and a rise of temperature (38.7° C.). Sedimentation rate was 120. During the past three weeks before his second admission to Department A of the University Hospital (Nov. 7, 1952) he had severe diarrhea, edema of the face and ankles, and albuminuria which had not been ascertained until October 31. Blood urea: 52 mg. per cent, serum protein: 3.26 per cent (albumin: 2.13, globulin: 1.13). The lymph nodes on the neck were only of pea size; there was no enlargement of the spleen. Edema, diarrhea, and severe albuminuria were indicative of amyloidosis. The patient died two weeks later, seventeen months after the onset of the disease.

In addition to the typical picture of Hodgkin's disease with involvement of mediastinal, bronchial, and paratracheal lymph nodes, the spleen, lungs, and the corpus of the fourth lumbar vertebra, the *autopsy* (record 331/52) showed a pronounced amyloidosis of the spleen, the kidneys, and the intestine.

Besides the changes in Hodgkin's disease with a pleomorphic cellular tissue with hyperchromatic Sternberg cells, *microscopic examination* showed a widespread amyloidosis in the previously mentioned organs, giving a positive amyloid reaction when stained with Congo red and the methyl violet stain for amyloid.

No conclusions as to the causal importance of Erasol to the development of amyloid can be drawn in this case but, considering the experimental findings reported, it seems probable that an inhibition of an active proliferation of mesenchymal cells caused by the treatment with nitrogen mustard has at least contributed to the formation of amyloid.

SUMMARY

In mice previously treated for several weeks with injections of sodium caseinate, three injections of nitrogen mustard (NH_2)—each equivalent to 2.5 to 5 mg. per kilogram of body weight—induced an almost diffuse amyloid deposition in the spleen.

There is evidence of *inhibition* of cellular proliferation in the reticuloendothelial system to account for amyloid formation induced by various means (cortisone, nitrogen mustard).

The concept is presented that suppression of plasma cells and other pyronine-positive cells, which are in a protracted stage of active proliferation, is a basic alteration during formation of amyloid.

A case of Hodgkin's disease in which an appearance of amyloidosis followed the treatment with nitrogen mustard and roentgen irradiation is reported.

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CHEMICAL SPECIFICITY OF DIABETOGENIC ACTION OF QUINOLINE DERIVATIVES

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NUMEROUS studies[1] have been made to find other diabetogenic chemical substances similar to alloxan, which are able to cause selective lesions of the pancreatic islet cells. Two groups of compounds—alloxan homologues,[2] some related compounds such as dehydroascorbic acid,[3] and some organic reagents[4, 5] (8-hydroxyquinoline, etc.)—have been shown to be diabetogenic. It is believed that similar destructive action on the islet cells by other compounds with different chemical composition may give a clue to the mechanism of action of these diabetogenic substances. Therefore, a study of the chemical properties and associated biological action would seem an important approach in determining the mode of action of these diabetogenic compounds.

Dunn, Sheehan, and McLetchie[6] reported that styrylquinoline No. 90 caused injury to the islets of Langerhans. Goldner and Gomori[7] have shown that quinoline and cincophen have no diabetogenic action, while Lukens and Kennedy[8] obtained similar results with several styrylquinoline derivatives. Recently, the diabetogenic action of 8-hydroxyquinoline was confirmed by Root and Chen,[9] but some quinoline compounds related to 8-hydroxyquinoline have been shown to have no diabetogenic action.

Little is known about the essential chemical structure of quinoline derivatives required for the diabetogenic action. Therefore, systematic studies on the diabetogenic action of quinoline derivatives structurally related to effective 8-hydroxyquinoline have been undertaken, with the hope that some chemical specificity in quinoline derivatives in relation to their diabetogenic action might be disclosed.

METHODS AND MATERIALS

Adult rabbits were employed. Blood sugar was determined by Hagedorn-Jensen's method.[10] For histologic examination of the pancreatic islet cells, Gomori's chromium-hematoxylin phloxine method[11] was used. Thirteen quinoline derivatives, of which constitutional formula, melting point, and injected doses are listed in Table I, were employed in this study. These were applied in solutions of dilute hydrochloric acid. Of these, quinaldine was a commercial product, and xanthurenic acid[12] was kindly supplied by Dr. Fred Rosen. All the others were synthesized by Dr. H. Horii who verified the structural character by standard analytical methods. The details of the methods of preparation of these compounds will be omitted in order to conserve space.

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Received for publication, Sept. 1, 1953.

TABLE I. QUINOLINE DERIVATIVES USED IN THIS EXPERIMENT AND OUTLINE OF RESULTS

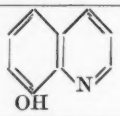
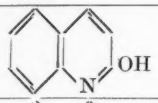
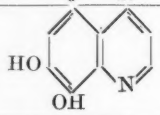
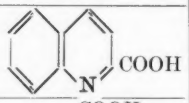
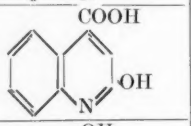
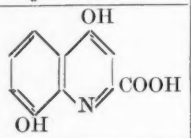
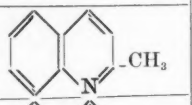
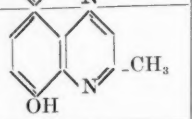
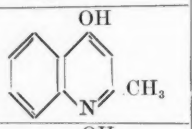
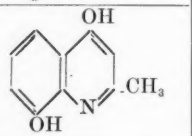
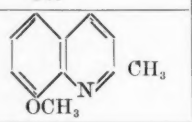
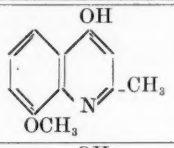
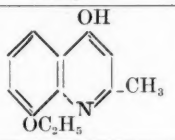
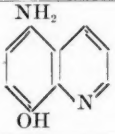
COMPOUNDS	STRUCTURAL FORMULA	MELTING POINT C.	INJECTED DOSES MG./KG.	EFFECT ON BLOOD SUGAR
8-Hydroxyquinoline (for comparison)		75°	50	Diabetogenic
2-Hydroxyquinoline		198°	50	No effect
7,8-Dihydroxyquinoline		282°-284°	40-500	No effect
Polyhydroxyquinoline		over 280°	100	No effect
Quinaldinic acid (for comparison)		155°-160°	100	No effect
2-Hydroxycinchoninic acid		346°	50-1,000	No effect
Xanthurenic acid		297°-298°	50-500	No effect
Quinaldine			7	No effect
8-Hydroxyquinaldine		74°-75°	40-50	Diabetogenic
4-Hydroxyquinaldine		231°	40	Occasionally temporary hyperglycemia
4,8-Dihydroxyquinaldine		259°-263°	50-100	No effect
8-Methoxyquinaldine		not purified	ca. 20	No effect

TABLE I—CONT'D

8-Methoxy-4-hydroxyquinaldine		222°	50-100	No effect
8-Ethoxy-4-hydroxyquinaldine		192°-193°	20-40	No effect
5-Amino-8-hydroxyquinoline			ca. 30-40	Diabetogenic

RESULTS

Action of Hydroxy-Compounds.—2-Hydroxyquinoline did not cause any remarkable change in blood sugar when compared with the same diabetogenic dose of 8-hydroxyquinoline; 7,8-Dihydroxyquinoline was less toxic and did not cause any change, even in doses up to 500 mg. per kilogram. Polyhydroxyquinoline was also less toxic and did not cause any fluctuation of blood sugar levels.

Action of Carboxyl Compounds.—Carboxyl compounds were far less toxic. Animals tolerated well injections of 2-hydroxy-cinchoninic acid and xanthurenic acid in doses respectively of 1,000 and 500 mg. per kilogram and at the same time they showed no alteration of blood sugar. After injection of xanthurenic acid, the animals excreted yellow-colored urine which showed a green color reaction with ferric ion.

Action of Quinaldine Derivatives.—In contrast to the above compounds, quinaldine derivatives were in general toxic. Among them, 8-hydroxyquinaldine was found to be diabetogenic.

Changes of Blood Sugar Caused by 8-Hydroxyquinaldine.—Changes of blood sugar in eleven rabbits after intravenous injection of 8-hydroxyquinaldine are shown in Table II and Fig. 1. Most of the rabbits showed transient hyperglycemia, which returned to normal values after twenty-four to forty-eight hours. Three rabbits showed hypoglycemia twenty-four hours after injection without showing hypoglycemic convulsions after which their blood sugar increased gradually to hyperglycemic levels. One rabbit maintained slightly hyperglycemic levels for six days after which the blood sugar returned to normal values. The other two rabbits continued hyperglycemic for two weeks and then recovered.

In the initial hyperglycemic phase, islets of Langerhans of rabbits, which were sacrificed four hours after injection, showed lesions in capillaries, decrease of beta granules, and pyknosis of their nuclei (Fig. 2). In the diabetic

TABLE II. CHANGES OF BLOOD SUGAR AFTER INTRAVENOUS INJECTION OF 8-HYDROXYQUINALDINE

RABBIT	BODY WEIGHT (GM.)	INJECTED DOSES (MG./KG.)	BLOOD SUGAR IN MG./100 C.C. BLOOD											
			BEFORE IN-INJECTION	1 HR.	2 HR.	4 HR.	8 HR.	24 HR.	48 HR.	3 DAY	4 DAY	5 DAY	6 DAY	7 DAY
1	2,700	40	94	218	256	271	224	140			82			
2	2,600	50	109	218	256	290	246	77		92				
3	3,000	50	100	108	146	167	75	45	305		347			299
4	2,500	50 (in NaOH)	85	217	244	179		174					87	
5	2,820	50	94	221	236	271		224				104		
6	2,300	50	112	191	230	303		116			118			
7	2,840	50	86	170	212	232	210	128		85				
8	2,640	50	119	258	276	282	231	44	194	176			103	
9	2,100	40	94	177	193	222	(sacrificed after 4 hr.)							
10	2,050	50	99	177	257	288	276	228			98			
11	2,000	50	103	178	258	251	245	38	274		260			

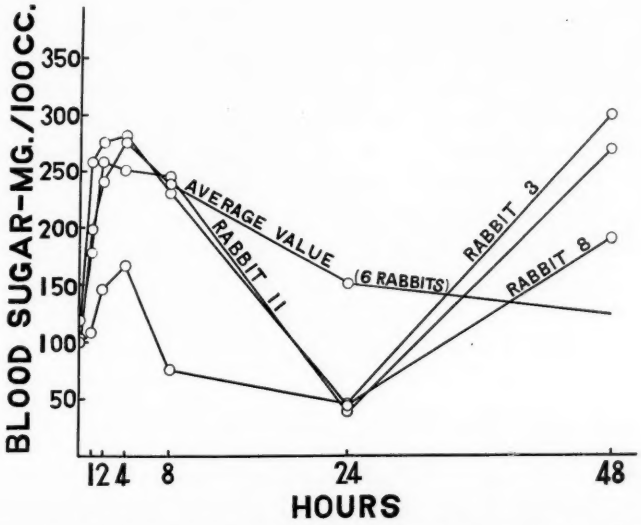


Fig. 1.—Changes in blood sugar levels in rabbits after intravenous injection of 40 to 50 mg. per kilogram of 8-hydroxyquinaldine.

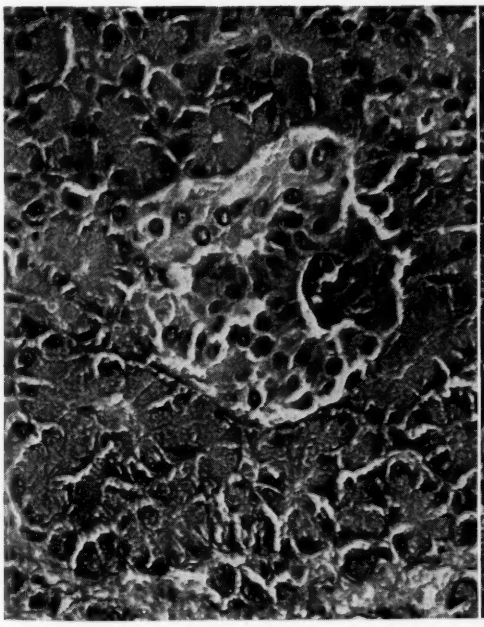


Fig. 2.

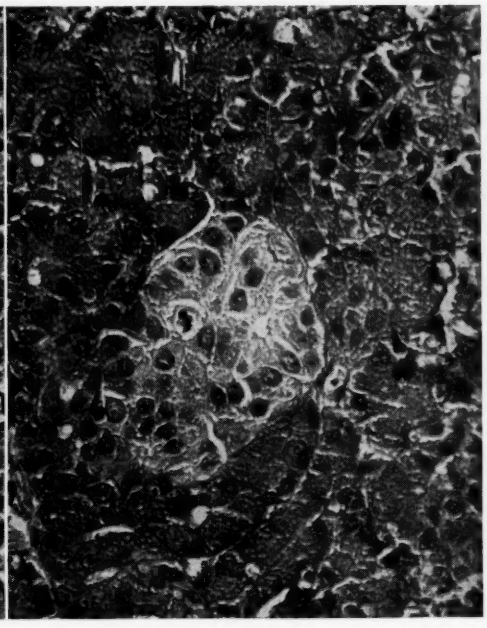


Fig. 3.

Fig. 2.—Islet of Langerhans of rabbit 9 which was sacrificed four hours after intravenous injection of 8-hydroxyquinaldine. Most of beta cells located at the periphery of the islet show decrease of their granules and nuclei of other beta cells are pyknotic. (Gomori's chromium-hematoxylin phloxine stain.) (Magnification ×500; reduced 1/6.)

Fig. 3.—Biopsy of islet of Langerhans from rabbit 3 at seven days which continued to be hyperglycemic for two weeks after intravenous injection of 8-hydroxyquinaldine. Islet is reduced in size and is chiefly composed of agranular cells and of a few alpha cells. Nuclei of agranular cells are enlarged and a mitotic figure can be seen. (Gomori's chromium-hematoxylin phloxine stain.) (Magnification ×500; reduced 1/6.)

TABLE III. CHANGES OF BLOOD SUGAR LEVELS AFTER INTRAVENOUS INJECTION OF 5-AMINO-8-HYDROXYQUINOLINE

RABBIT	BODY WEIGHT (GM.)	INJECTED DOSES (MG./KG.)	BLOOD SUGAR IN MG./100 C.C. BLOOD										
			BEFORE INJECTION	1 HR.	2 HR.	4 HR.	24 HR.	48 HR.	3 DAY	5 DAY	7 DAY	10 DAY	14 DAY
5A-1	2,390	ca. 30	127	151	161	207	78	187	236	228	255	168	195
5A-4	3,400	ca. 30	124	205	(died after 15 minutes)								
5A-5	2,620	ca. 30	119	106	128	166	75	122	113				
5A-11	2,440	ca. 40	103	278	265	144	71	67	79				
5A-12	2,960	ca. 40	113	207	203	195	205 (8 hr.)	(died after 8 hr.)					
5A-13	2,740	ca. 40	85	330	(died after 1 hr.)								
5A-15	2,400	ca. 40	106	169	185	176	139	90					
5A-17	2,480	ca. 40	95	148	210	134	93						
5A-18	2,960	ca. 40	146	196	200	155							
5A-20	2,960	ca. 40	107		235	131	(died after 4 hr.)						
5A-23	2,420	ca. 40	104	125	125	125	70	146		114	277	198	
5A-24	2,900	ca. 40	137	225	240	197	146	166		239	351	355 (8 days) (died after 8 days)	
5A-25	2,420	ca. 40	95	151	132	118	75	98		87	100		
5A-26	2,280	ca. 40	115	131	171	149	(died after 3 hr.)						
5A-27	2,680	ca. 40	98	168	(died after 20 minutes)								

phase after seven days, the islets were reduced in number and size and were mostly composed of alpha cells and agranular cells with large nuclei. Occasionally, mitotic figures were observed in the latter cells (Fig. 3).

Action of 4-Hydroxyquinaldine.—This compound caused severe convulsions with doses of 40 to 50 mg. per kilogram, but the animals survived. No fluctuation of blood sugar was observed, except in only a few cases which showed a temporary hyperglycemia. A few rabbits showed a hyperglycemia for one to two weeks after injection after which the blood sugar values returned to normal. Histologic examination of the islets from animals which died soon after injection revealed no remarkable changes. Pyknosis of nuclei was observed in the beta cells in some animals which maintained a hyperglycemia for several days.

4, 8-Dihydroxyquinaldine did not cause any change of blood sugar levels in spite of its considerable toxicity. This compound appeared to be less toxic than the former two compounds and its lethal dose range varied from 150 to 200 mg. per kilogram. Other quinaldine derivatives, 8-methoxyquinaldine, 8-methoxy-4-hydroxyquinaldine, 8-ethoxy-4-hydroxyquinaldine failed to produce any fluctuation in blood sugars with doses used in this experiment.

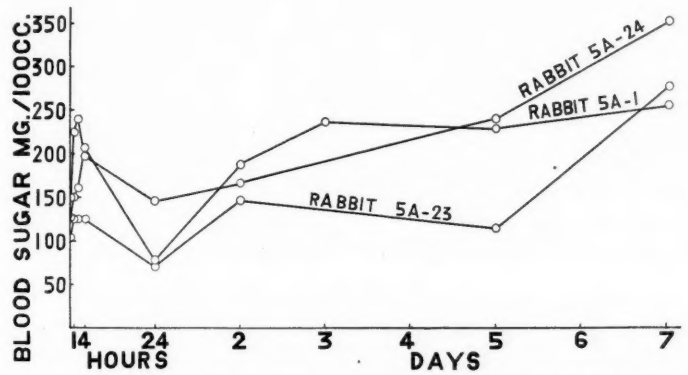


Fig. 4.—Changes of blood sugar after intravenous injection into rabbits of approximately 30 to 40 mg. per kilogram of 5-amino-8-hydroxyquinoline.

Action of Amino-Compounds: Changes in Blood Sugar Levels Caused by 5-Amino-8-Hydroxyquinoline (5-Amino-Oxine).—Changes of blood sugar levels in fifteen rabbits after intravenous injection of 5-amino-8-hydroxyquinoline are shown in Table III and Fig. 4. This compound showed considerable toxicity. Most rabbits showed transient hyperglycemia, which returned to normal value after twenty-four hours. Six rabbits died after fifteen minutes to eight hours with hyperglycemia. Having shown a slight hypoglycemia at twenty-four hours, the blood sugar of the other three rabbits increased gradually after forty-eight hours and continued at hyperglycemic levels for seven to eight days.

In the initial hyperglycemic phase (Rabbit 5A-27, 5A-13) the islets showed marked alteration in the capillaries and a decrease of beta granules with slight destruction of beta cells (Fig. 5). Islets of rabbits which died

after three to eight hours showed slight or moderate destruction of capillaries and decrease of beta granules, accompanied by marked destruction of beta cells in one rabbit (5A-20) (Fig. 6). In the secondary hyperglycemic phase after seven to eight days, islets were reduced in number and size and were composed of agranular beta cells which often contained vacuoles. Usually alpha cells showed no changes but in one rabbit they appeared to be decreased in number (5A-1).

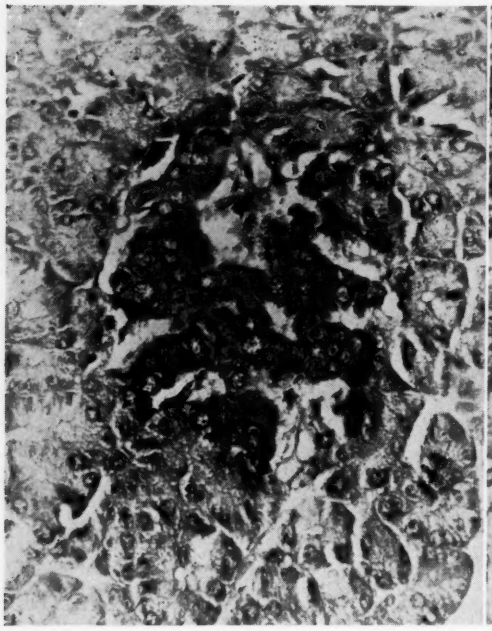


Fig. 5.

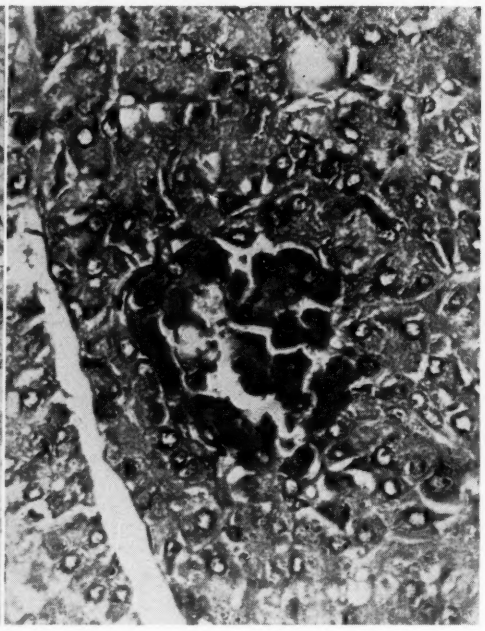


Fig. 6.

Fig. 5.—Islet of Langerhans of rabbit 5A-13, which died one hour after intravenous injection of 5-amino-8-hydroxyquinoline. Islet shows capillary lesions and widening of cell cords. Most of beta cells show decrease of their granules and a few large nuclei appear. (Gomori's chromium-hematoxylin phloxine stain.) (Magnification ×500; reduced ⅙.)

Fig. 6.—Islet of Langerhans of rabbit 5A-20, which died four hours after intravenous injection of 5-amino-8-hydroxyquinoline. Center of islet is destroyed and a necrotic mass with a few pyknotic nuclei remains. Alpha cells appear normal. (Gomori's chromium-hematoxylin phloxine stain.) (Magnification ×500; reduced ⅙.)

DISCUSSION

As has already been reported,[4, 5] it was confirmed that 8-hydroxyquinoline (oxine) and some organic reagents in suitable doses caused diabetes in rabbits by destroying pancreatic islet cells. These compounds were shown to react selectively with zinc in islet cells which are considered to be intimately concerned with insulin synthesis. The mechanism of action of these organic reagents in producing diabetes is not completely known although previous studies indicate that the diabetogenic action of these compounds is associated with their toxic nature and close affinity for various metals.

Present studies have shown that certain chemical structures in quinoline derivatives are essential for eliciting a diabetogenic reaction. It is clear that monohydroxyquinoline (other than in position 8) caused no effect. The in-

crease of hydroxy groups in 8-hydroxyquinoline decreased remarkably their toxicity and abolished the diabetogenic activity. Carboxyl compounds were proved to be less toxic. Large doses, such as 1,000 mg. per kilogram of 2-hydroxy-cinchoninic acid and 500 mg. per kilogram of xanthurenic acid, were tolerated and caused no effect on blood sugar. Xanthurenic acid[13] was first isolated from the urine of a pyridoxine-deficient rat, fed with massive doses of tryptophane. It was shown that this acid originated from the dietary tryptophane. Recently, Kotake and Inada[14] reported that rats which excrete xanthurenic acid also show glycosuria. Furthermore, they showed that xanthurenic acid isolated from the urine of these rats, when injected intraperitoneally in doses of 120 mg. per kilogram into normal rats, elicited a diabetic reaction.

Our studies show that xanthurenic acid is a nontoxic substance, that it does not cause any effect on blood sugar, and that it was excreted in the urine soon after injection. Miller and Baumann[15] also reported that 50 to 58 per cent of xanthurenic acid injected intraperitoneally was excreted in the urine within twenty-four hours. It is questionable as to whether the xanthurenic acid used by Kotake and Inada was actually this compound for it was not shown to be on chemical analysis. Furthermore, their results are open to question because all previous diabetogenic agents are toxic substances, and the toxic doses are somewhat more than the diabetogenic doses. In addition, available evidence shows clearly that increasing the hydroxy or carboxyl groups in toxic diabetogenic quinoline compounds (which have hydroxy groups in position 8) greatly diminishes their toxicity and abolishes the specific diabetogenic effect. Nontoxicity of xanthurenic acid is also presumed from the fact that kynurenic acid which originates from tryptophane is nontoxic and has no biologic action.

Although quinaldine has no diabetogenic action, despite its marked toxicity, 8-hydroxyquinaldine was proved to be diabetogenic as is 8-hydroxyquinoline; 8-hydroxyquinaldine[16, 17] is also reported to be an organic reagent which reacts with various metals but shows some differences to 8-hydroxyquinoline in its reaction with aluminum. This agent caused temporary hyperglycemia in most animals. Occurrence of subsequent hypoglycemia in a few rabbits may be attributed to the variation in reaction of the animals. During the hypoglycemic phase caused by 8-hydroxyquinaldine, no convulsions were observed and subsequent hyperglycemia was slight. This might be interpreted as the recovery of insulin synthesis due to vigorous regeneration of islet beta cells after destruction, as shown by their frequent mitotic figures. Substitution of the hydroxy group with the methoxy group in position 8 rendered the compound inactive. This fact agrees with Root and Chen's report[9] that 8-methoxyquinoline has no diabetogenic action. While the present studies were in progress the diabetogenic action of 8-hydroxyquinaldine in rabbits was also shown by Okamoto.[18]

4-hydroxyquinaldine was found to be toxic similarly to 8-hydroxyquinaldine, but it has no diabetogenic action. A slight temporary hyperglycemia

and hyperglycemia after several days were observed in a few cases. Why this occurred is not clear. Increase of a hydroxy group in position 4 in diabetogenic 8-hydroxyquinaldine rendered the compound inactive. It is, however, of interest to note that the toxicity of these two compounds is not markedly reduced as in the case of other quinoline derivatives whose lethal doses are three to four times those of 8-hydroxyquinaldine and 4-hydroxyquinaldine. Similar compounds, 8-methoxy-4-hydroxyquinaldine and 8-ethoxy-4-hydroxyquinaldine, also were proved to be inactive.

Addition of an amino group in position 5 to 8-hydroxyquinoline causes no change in diabetogenic action. This compound was markedly toxic and showed a diabetogenic action similar to 8-hydroxyquinoline and 8-hydroxyquinaldine. Secondary hyperglycemia caused by 5-amino-8-hydroxyquinoline occurs after three to seven days and seems to appear somewhat later than that produced by other diabetogenic reagents.

SUMMARY

The diabetogenic action of thirteen quinoline derivatives structurally related to 8-hydroxyquinoline was examined in rabbits. Close correlation between chemical structure and diabetogenic action in quinoline compounds has been established; 8-hydroxyquinaldine and 5-amino-8-hydroxyquinoline were confirmed to be diabetogenic, but 8-methoxyquinaldine was not. A hydroxy group in position 8 of quinoline was essential for diabetogenic action. The addition of hydroxy and carboxyl groups abolished the specific effect which was associated with a decrease in the toxicity. Since diabetogenic 8-hydroxyquinaldine and 5-amino-8-hydroxyquinoline have properties of an organic reagent, a close correlation between destructive action of the pancreatic islet cells and binding properties with metal ions was confirmed. The ability of these compounds to bind metal ions may explain their cytotoxic effect and thus their diabetogenic action. Although other possibilities exist to explain the specificity of these diabetogenic compounds the available evidence shows clearly that their diabetogenic action must be intimately associated with their ability to react sensitively and selectively with trace metals. Much more experimental work must be done before a clear picture of the essential steps in the production of diabetes by these compounds is elicited.

We are greatly indebted to Dr. F. Rosen for the generous supply of xanthurenic acid and to Dr. H. Horii for the synthesis of all other compounds used in this study. We are also indebted to Dr. K. K. Chen for his kind instruction on references. The photomicrographs were made by Mr. R. Tada.

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THE HEMATOLOGIC RESPONSE TO ADRENALIN

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BROOKLYN, N. Y.

INTRODUCTION

AN ELEVATION of the leukocyte count following the injection of Adrenalin into both normal human subjects[1, 2, 3] and patients with various disease states[4, 5] has been repeatedly observed. Changes in erythrocyte[6, 7] and platelet levels[8, 9] have not been as constant a finding.

Doan and Wright[5] have applied the Adrenalin "test" to the study of patients with the "hypersplenic syndrome." They have considered the response in these patients to be specific, involving only the depressed element(s) and have correlated this with the view that the original depression is caused by abnormal splenic sequestration. It is postulated that the effect of Adrenalin is primarily on the spleen, causing it to contract, thereby squeezing the sequestered cells into the peripheral circulation. Confusion has arisen since other observers[10, 11] have not found an abnormal response in patients with "hypersplenism." In addition, it has been shown that splenectomized patients[4] manifest essentially normal leukocyte changes following Adrenalin injection.

It is the purpose of this report to present the hematologic effects noted after the administration of Adrenalin to a group of patients with normal hemograms and various hematologic disorders. An attempt has been made to determine whether a correlation exists between the type of changes seen in the formed elements of the blood and the presence of any of the pathologic states studied.

METHODS

Forty-seven Adrenalin tests were performed on 35 patients. All studies were made in the morning while the patient was resting but not necessarily under basal condition; 0.5-1.0 ml. of 1/1,000 solution of Adrenalin Chloride was injected intramuscularly. The intramuscular site was chosen to conform with previous observations in normal human subjects.[1] Two control studies were made at ten-minute intervals prior to Adrenalin injection and test samples were taken at periods of five, fifteen, and thirty minutes as well as one, two, three, and four hours after the injection. The determinations of the formed elements in all cases were made on capillary blood. White cells were counted in routine fashion in duplicate. Differential white counts were determined from 200 cells on cover slip smears stained with Wright's stain. Hemoglobin determinations were made photocolorimetrically using ferric cyanide. Platelet counts were performed according to the method described by Dameshek.[12]

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This work was presented in abstract at the National Meeting of the American Federation for Clinical Research, May 4, 1952.

Received for publication, June 19, 1953.

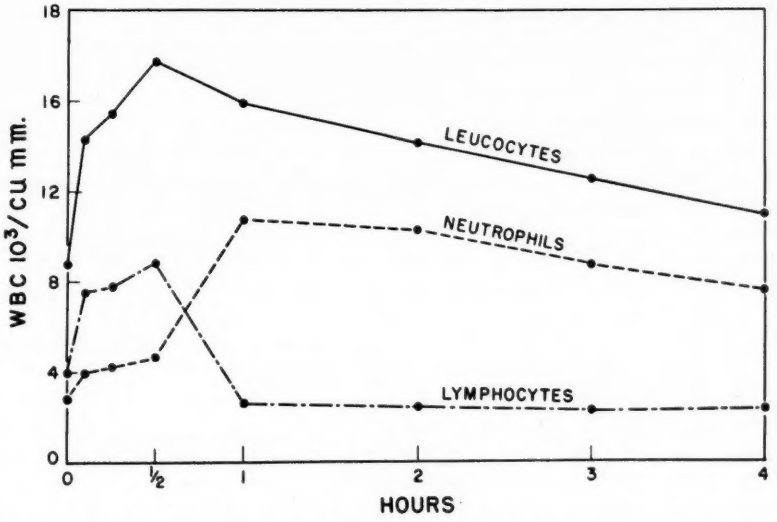

Fig. 1.—Leukocyte response in patient G. V. following the intramuscular injection of 1 ml. of 1:1,000 Adrenalin Chloride. The curve in general is representative of most of the cases studied.

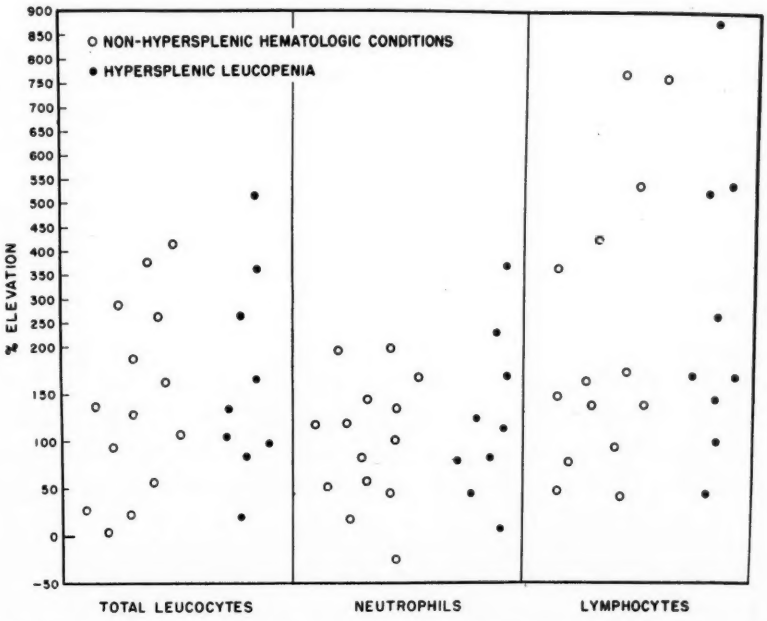

Fig. 2.—The maximal per cent change of leukocytes in the first hour following Adrenalin injection in a group of patients with hypersplenic and nonhypersplenic hematologic disorders. Each circle indicates an individual case (Table I), and represents the maximal per cent change from base-line levels.

The 35 patients (Table I) included the following categories: Cooley's anemia, 8; congestive splenomegaly, secondary to cirrhosis of the liver, 7; sickle-cell anemia, 3; aplastic anemia, 3; polycythemia vera, 2; and 1 each of myelofibrosis with myeloid metaplasia, myeloblastic leukemia, and acquired hemolytic anemia. Nine patients had normal hemograms (Addison's disease, 3; and 1 each of pulmonary emphysema, lung abscess, rectal fissure, Cushing's syndrome, sciatica, and cirrhosis of the liver).

The systemic manifestations were noted in each patient. The degree and the time of maximal splenic contraction were carefully observed by frequent palpation of the abdomen. All of the patients with hematologic syndromes had extensive clinical studies which included bone marrow examinations. Of this group, 21 patients had splenomegaly. Eleven of them were considered to have "secondary hypersplenism." This diagnosis was presumed when there was a peripheral cytopenia and splenomegaly associated with normal or increased presence of the depressed element(s) in the bone marrow. It was confirmed by a favorable response to splenectomy when performed (five cases). The primary diagnosis in seven of the "hypersplenic" patients was congestive splenomegaly associated with cirrhosis of the liver. Five of these patients had pancytopenia and two leukopenia and anemia. In four patients the primary diagnosis was Cooley's anemia. All four had leukopenia and one patient had in addition thrombopenia.

The patients with Cooley's anemia, in addition to showing leukopenia which is unusual for this condition,[13] also significantly revealed an abnormal rate of destruction of transfused normal red cells as measured by Ashby survival curves.[14] These patients also required an exceptional number of transfusions and for all these considerations six of them (3 studied here) were splenectomized. In all, the hemolytic component was ameliorated following splenectomy and the leukocyte count reverted to normal.

A total of nine splenectomized patients was investigated; six patients were studied before and after operation and in three, only postsplenectomy studies were done.

RESULTS

General Adrenalin Response.—In each patient studied, there was a prompt increase of all white cell elements in the first hour (Phase 1). This was similar to the response previously reported for normal subjects.[1] The peak elevation of total leukocytes occurred between five and thirty minutes and was not necessarily related to the time of maximal splenic contraction. Immature cells were not noted except in cases where these were present before injection. All the patients manifested one or more of the features typical of increased sympathomimetic activity.

In the second phase (two to four hours), the neutrophils continued to remain above the base-line levels while the lymphocytes fell below their preinjection values. In contrast to the general response reported for normal subjects, the neutrophils did not show a continuous rise throughout the second phase in most cases, and therefore in these patients a biphasic curve for total leukocytes was not obtained (Fig. 1).

Adrenalin Response in Subjects with "Nonhypersplenic" Hematologic Conditions.—As illustrated in Fig. 2, there was a wide scatter of leukocyte response to Adrenalin injection in the group of patients with "nonhypersplenic" hematologic conditions. All of these patients with the exception of three with aplastic anemia had splenomegaly. Responses are given as the maximal per cent change above the base-line levels. The three patients who manifested the smallest percentage increase in cells all had an initial leukocytosis.

TABLE I. LEUKOCYTE RESPONSE* TO ADRENALIN IN VARIOUS CLINICAL STATES
INDIVIDUAL AND MEAN CHANGES

	PATIENT DIAGNOSIS	BASE-LINE WHITE COUNT PER CUBIC MILLIMETER	TOTAL WHITE COUNT PER CUBIC MILLIMETER		NEUTROPHILS PER CUBIC MILLIMETER		LYMPHOCYTES PER CUBIC MILLIMETER	
			ABSOLUTE	RELATIVE (%)	ABSOLUTE	RELATIVE (%)	ABSOLUTE	RELATIVE (%)
	Individual changes							
J. D.	Sickle-cell anemia	12,050	3,350	28	2,821	51	2,489	50
P. H.	Sickle-cell anemia	16,225	4,075	24	1,904	20	2,560	48
F. M.	Sickle-cell anemia	5,750	7,800	136	4,668	119	541	145
D. C.‡	Cooley's anemia	2,794	4,125	188	849	198	2,746	154
H. P.‡	Cooley's anemia	3,438	9,262	269	3,568	170	5,762	525
S. A.	Cooley's anemia	8,100	7,750	96	3,554	84	2,750	80
C. A.	Cooley's anemia	7,600	9,900	109	6,197	159	2,758	202
F. L.‡	Cooley's anemia	4,825	17,400	361	5,752	222	10,653	529
M. G.	Cooley's anemia	7,300	9,200	126	5,352	141	4,890	173
L. I.‡	Cooley's anemia	2,150	11,000	511	3,463	363	6,877	879
C. N.	Polycythemia vera	18,238	9,637	53	7,913	51	1,705	96
H. S.	Polycythemia vera	17,800	650	4	-3,522	-23	2,116	359
R. L.	Aplastic anemia	4,575	11,675	255	1,341	114	9,654	422
E. S.	Aplastic anemia	1,925	7,285	372	1,505	195	5,205	768
E. R.	Aplastic anemia	1,800	7,300	406	278	61	6,903	536
M. N.	Myelofibrosis	4,100	11,725	286	2,849	103	7,737	764
A. M.	Acquired hemolytic anemia	6,350	10,275	161	5,780	168	2,877	148
K. N.	Myeloblastic leukemia	39,000	42,000	107	8,270	131	8,550	164
O. S.‡	Congestive splenomegaly†	2,750	3,660	131	808	46	1,004	148
J. S.‡	Congestive splenomegaly†	3,950	6,350	160	2,891	112	2,914	254
F. A.‡	Congestive splenomegaly†	4,827	3,920	81	2,105	69	1,761	101
M. N.‡	Congestive splenomegaly†	3,950	4,175	105	2,423	121	2,273	166
G. B.‡	Congestive splenomegaly†	2,900	425	14	-227	-45	347	38
B. G.	Addison's disease	6,520	6,000	92	1,837	41	4,558	244
D. S.	Addison's disease	7,250	11,400	157	3,420	99	8,057	250
J. C.	Addison's disease	7,100	6,775	95	1,732	65	4,592	161
M. L.	Pulmonary emphysema	9,125	4,225	46	1,487	24	1,938	75
F. P.	Lung abscess	5,927	6,066	102	1,718	44	6,345	270
A. G.	Rectal fissure	6,200	6,812	111	2,331	65	3,072	100
C. S.	Cushing's syndrome	8,200	7,325	89	4,881	79	1,931	111
S. L.	Sciatica	8,290	6,850	83	2,662	40	2,607	220
J. G.	Cirrhosis of liver	7,688	6,647	86	4,145	87	2,365	102
	Mean Changes							
(9)	Patients with normal leukocyte levels	7,322	6,900	96	2,691	60	3,885	170
(11)	Patients with splenomegaly and normal or increased leukocyte levels§	10,237	10,578	103	4,162	91	3,543	203
(3)	Patients with aplastic anemia	2,433	8,753	344	1,041	123	7,254	575
(9)	Patients with "Hypersplenic Leukopenia"	3,509	6,702	202	2,403	140	3,815	310

*Response is given as maximal absolute and per cent change from contral levels during the first hour. In many cases the maximum neutrophil and lymphocyte change did not occur at the same time (e.g., Fig. 1). Therefore, the arithmetical sum of these individual levels does not necessarily correspond exactly to the maximum total white count. In addition there were small but not distinctive changes in the other white blood cells.

†Secondary to cirrhosis of liver.

‡Patients with "hypersplenic leukopenia."

§One patient (M. N.) in this arbitrary grouping had a leukocyte level below normal. All leukocyte counts of patients with Cooley's anemia are corrected for normoblasts. The results listed above do not include any postsplenectomy studies (Table II).

There was a similarly wide range when the responses were judged in terms of absolute increase rather than percentage increase of cells (Table I). The range of increase for mature leukocytes was 650 to 16,820 per cubic millimeter, for neutrophils 280 to 8,270 per cubic millimeter, and lymphocytes 541 to 9,600 per cubic millimeter.

The mean response of this group (10,578 cells per cubic millimeter) was higher than the mean elevation (6,900 cells per cubic millimeter) of the group with normal leukocyte levels. One patient with myeloblastic leukemia manifested the highest total leukocyte rise, largely due to a marked increase of immature cells.

There was no distinctive pattern of leukocyte changes in any of the groups studied except in the instance of aplastic anemia. Each of the three patients with this condition revealed a marked percentage increase of total leukocytes, due to a high relative (mean 575 per cent cells per cubic millimeter) and absolute (mean 7,524 cells per cubic millimeter) rise of lymphocytes. The absolute neutrophil increase was much lower than average.

Adrenalin Response in Subjects with "Secondary Hypersplenic Leukopenia."—In Fig. 2, the per cent responses of the patients with "hypersplenic leukopenia" are compared with the results in other conditions. There was a wide scatter of leukocyte response in these patients which was similar to the other hematologic conditions. No distinction is made on this chart between the patients with Cooley's anemia and leukopenia and those with congestive splenomegaly and leukopenia, since we believe, for reasons stated previously, that they both represent valid examples of secondary "hypersplenic leukopenia." Realizing, however, that the syndrome in Cooley's anemia is unusual, individual responses are listed in Table I.

The absolute cell changes in the members of this group also varied within a wide range which could not be distinguished from the scatter of the "nonhypersplenic" patients.

Effect of Splenectomy on the Results of the Adrenalin Test.—Six patients, three of whom had "hypersplenic leukopenia," were studied before and after splenectomy (Table II). Three other patients, two of whom had had "hypersplenic leukopenia," were studied only after operation. Splenectomy improved the hematologic status of each of the patients.

The range of the response in splenectomized subjects was like that observed among other groups, for leukocytes 3,375 to 18,000; for neutrophils 1,177 to 11,000 and lymphocytes 571 to 14,700.

Of the patients studied before and after splenectomy, two had similarly low percentage changes before and after operation. The other four patients showed a distinct percentage drop in response to Adrenalin after removal of the spleen. These patients all had markedly higher base-line white cell counts at the time of the postoperative study than at the time of the preoperative one. A comparison of their Adrenalin response in terms of absolute increase of cells, however, showed very similar total leukocyte changes before and after splenectomy in three of these four patients.

In two patients, the postoperative study was repeated once again when there was a further change in the leukocyte count. The absolute change in the presplenectomy and postsplenectomy studies was again found to be very close. Since the base-line leukocyte count differed in each study, the proportionate change was found to vary inversely with the initial leukocyte level.

TABLE II. LEUKOCYTE RESPONSE* TO ADRENALIN BEFORE AND AFTER SPLENECTOMY

PATIENT	DIAGNOSIS	SPLENECTOMY	BASE-LINE WHITE COUNT PER CUBIC MILLIMETER	TOTAL WHITE RESPONSE PER CUBIC MILLIMETER		NEUTROPHIL RESPONSE PER CUBIC MILLIMETER		LYMPOCYTE RESPONSE PER CUBIC MILLIMETER	
				ABSOLUTE	RELATIVE (%)	ABSOLUTE	RELATIVE (%)	ABSOLUTE	RELATIVE (%)
H. P.‡	Cooley's anemia	Before	3,438	9,262	269	3,568	170	5,763	525
		After	11,500	10,575	92	10,383	147	3,711	150
			15,560	11,390	73	11,476	108	5,680	146
F. L.‡	Cooley's anemia	Before	4,825	17,400	361	5,752	222	10,653	529
		After	14,200	18,300	128	7,286	82	9,612	271
C. A.	Cooley's anemia	Before	5,300	9,900	188	6,197	198	2,758	154
		After	28,000	5,640	19	1,177	5	2,650	86
V. S.	Cooley's anemia	Before	—	—	—	—	—	—	—
		After	33,000	15,100	46	3,962	35	11,900	76
J. D.	Sickle-cell anemia	Before	12,050	3,350	28	2,821	51	2,489	49
		After	10,925	3,525	33	4,954	90	571	23
			22,238	4,812	22	2,052	14	4,002	71
G. B.‡	Congestive splenomegaly†	Before	2,900	425	14	-227	-45	347	38
		After	8,950	3,375	38	2,868	40	1,424	187
G. V.‡§	Congestive splenomegaly†	Before	—	—	—	—	—	—	—
		After	8,713	8,762	101	8,111	291	4,999	130
K. N.‡§	Congestive splenomegaly†	Before	—	—	—	—	—	—	—
		After	34,313	18,112	56	8,060	48	14,756	136
A. M.	Hemolytic anemia	Before	6,300	10,275	161	5,780	168	2,877	137
		After	11,700	10,700	91	4,591	87	4,942	100

*Maximal absolute and per cent change from base-line levels during the first hour.
†Secondary to cirrhosis of liver.
‡Patients with "hypersplenic leukopenia."
§These patients had "hypersplenic pancytopenia" before operation. Preoperative Adrenalin tests were not done.
All white counts for the patients with Cooley's anemia are corrected for normoblasts.

The respective changes in neutrophils and lymphocytes did not show as close similarity as for total leukocytes.

Second Phase Adrenalin Response.—The second phase response in all conditions studied was similar to the normal response, except for the neutrophil curve already mentioned. There was no difference discernible in the second phase response of "hypersplenic" patients compared to those with other hematologic conditions.

Since the lymphopenia of the second phase has been attributed to adrenal cortical stimulation,[15] it was of interest that the three patients with Addison's disease nevertheless manifested a distinct drop in lymphocytes in this period. Their mean drop was 46 per cent as compared to a group of normals with a mean decrease of 15 per cent.

Response of Hemoglobin and Platelets to Adrenalin.—Hemoglobin determinations performed after the administration of Adrenalin disclosed a variable increase up to 3 Gm. per 100 ml. of blood. No distinctive pattern was noted,

either in the presence of "hypersplenic anemia" or relative to whether the test was done before or after splenectomy.

Platelet studies were done on twelve patients—six with thrombocytopenia and six with normal levels. With one exception the changes noted were not considered significant. There was a maximal increase of platelets of 150,000 in one patient who had myelofibrosis and myeloid metaplasia.

DISCUSSION

The mechanisms involved in the general leukocyte response to Adrenalin have been under consideration for a long period of time. Theories of hemoconcentration, and direct bone marrow stimulation have already been reviewed and refuted by other authors.[3, 4] Contraction of the spleen is a frequent occurrence after Adrenalin injection, but it would seem from this and other studies[16] that the splenic contribution to the over-all leukocyte response is not very great. The response in splenectomized subjects is essentially within the same range as the response of normals and of patients with splenomegaly. There is little difference for the individual patient in absolute leukocyte change before and after splenectomy.

The most widely postulated[2, 17] theory of leukocyte response to Adrenalin is that based upon a redistribution of cells from peripheral and visceral sources. Adrenalin increases cardiac output, with a resultant increase in velocity of blood flow, at the same time it dilates most peripheral blood vessels and constricts those in most viscera.[18, 19] Cells lying immobile in the capillary beds of many tissues and organs would then be swept into active circulation. The relative contribution of each organ has not as yet been accurately estimated. An important role of the lung has been suggested by the work of Bierman and co-workers[8, 20] who have demonstrated a greatly augmented leukocyte and platelet outpouring from the lung after Adrenalin injection. It is interesting that they detected the platelet increase only in arterial blood and were unable, like ourselves, to find any significant change in most venous blood samples. The augmented lymphocyte response in the first hour is probably due to the additional effect of lymph node contraction and increased thoracic lymph flow superimposed on the redistribution of lymphocytes from peripheral sources.

It would appear that additional factors related to adrenal function are operative in Phase 2 to produce the lymphopenia. Adrenalin has been shown to produce a lymphopenia two to four hours after injection in normal human subjects.[21] This lymphopenia occurs in the absence of the pituitary, but the response will not occur in the absence of the adrenal gland.[22] These observations indicate that the production of a lymphopenia by Adrenalin is dependent on the presence of functioning adrenal tissue. However, Gabrilove and co-workers[23] have shown that patients with Addison's disease do show a lymphopenic response only slightly less than normal after Adrenalin injection. We have observed a similar response, but in our patients with Addison's disease, the lymphocyte depression was even greater than normal. Apparently, then, the lymphopenic action of Adrenalin is dependent on the presence but not necessarily complete function of adrenal tissue.

The leukocyte response of the first hour has received most hematologic application. This initial rise which occurs in normal persons as well as in the patients with varied hematologic disorders, would appear to be a physiologic reaction, as described, dependent in part upon the availability of cells in all the tissues. This seems to be suggested by the low neutrophil responses in patients with aplastic anemia. It is apparently not related to abnormal splenic function nor to any other factor which may be involved in the production of the secondary "hypersplenic syndrome." The patients in this and other groups, with the exception of aplastic anemia, did not reveal any distinctive hematologic alterations after Adrenalin injection.

In the differential diagnosis of leukopenia in association with splenomegaly a "positive" Adrenalin test would not necessarily implicate "hypersplenism." However, it is conceivable that a low neutrophil response may be of aid in the diagnosis of disorders with primary bone marrow depression.

SUMMARY

Adrenalin was administered to 35 patients, 26 of whom had diverse hematologic conditions, including 21 with splenomegaly. The only condition showing distinctive changes was aplastic anemia where the neutrophil response was decreased and the lymphocyte increase marked. No diagnostic features were shown by 9 patients with secondary "hypersplenic leukopenia."

No characteristic changes occurred in hemoglobin or platelets in any of the cases studied.

Patients studied after splenectomy showed a rise of total leukocytes comparable to normal persons. Patients studied before and after splenectomy showed essentially similar responses.

It is postulated that the early leukocyte rise is due to a redistribution of cells swept into circulation from organs and tissues as a result of the vasomotor action of Adrenalin.

We wish to express our deepest appreciation to the late Dr. Emanuel B. Schoenbach and to Drs. R. Janet Watson and Herbert Lichtmen for their constant encouragement and guidance. We also wish to thank Misses Naomi Singer, Kathryn Wells, and Jean Robinson for their technical assistance, and Miss Eileen Shefner for her secretarial aid.

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ADDENDUM

Since the completion of this manuscript, a similar study confirming many of the present findings has been published by Chatterjea, J. B., Dameshek, W., and Stefanini, M., in Blood **8**: 211, 1953.

CORRELATION BETWEEN CAPILLARY RESISTANCE AND CIRCULATING EOSINOPHILS

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THE significance of the capillary resistance has increased considerably from both the theoretical and the practical points of view in the last few years. Previously it was looked upon exclusively as an indicator of the capacity of the capillary wall to resist increased intracapillary pressure or extracapillary suction, and the various factors capable of influencing this capacity were thought to exert their effects locally. Recent clinical observations[1, 2] and experimental work[3, 4] suggest that capillary resistance is not only a local phenomenon but is subject to hormonal regulation. The adrenal cortex is principally* involved and cortisone is its mediator.

Since the level of the circulating eosinophils, or better, its change upon induced cortical activation,[5] is considered to be a reliable and feasible indicator of adrenocortical activity, it was decided to study the correlation between the capillary resistance and the eosinophils and to compare their respective values in the assessment of adrenocortical function. The following experimental conditions were chosen for this study: (1) Adrenalectomy in the normal rat. (2) Administration and withdrawal of cortisone in the adrenalectomized rat. (3) Prolonged administration of cortisone in the adrenalectomized rat and dog. (4) Prolonged administration of cortisone to the normal dog. (5) Forced muscular exercise in the normal rat. (6) Cold stress in the normal rat. (7) Prolonged fasting in the normal dog.

In the first four groups of experiments a change in the cortisone level was elicited and its effect studied. In addition, these experiments gave us an opportunity to follow the prolonged effect of a constantly low (Experiments 1 and 2) and a constantly high (Experiments 3 and 4) concentration of cortisone in the body.

The stress experiments (Nos. 5, 6, and 7) were planned with these previous findings in mind. It has been demonstrated that various forms of stress (surgical trauma, anesthesia, muscular exercise, cold) when applied in a sufficient degree to the albino rat elicit a typical response in the capillary resistance in the majority of animals which consists of four phases.[4, 6] The first phase, the initial rise of the resistance immediately following the stress and last-

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This work represents part of a project supported by the Cardiovascular Section of the United States Public Health Service and by The Creighton University School of Medicine.

Received for publication, Aug. 10, 1953.

*On the basis of work in progress it appears that the somatotropic hormone of the anterior pituitary also participates in the regulatory mechanism.

ing for 1 to 5 days is a recognized phenomenon following trauma[7, 8] as well as in other forms of stress.[2] This initial rise, however, is followed with great regularity by a precipitous drop in the resistance (second phase), whereafter the capillary resistance remains at pathologically low levels for several days or even weeks (third phase) until, in case of survival, a gradual restoration takes place (fourth phase). By testing the animals with ACTH and cortisone it was demonstrated that the second and third phases, termed "capillary crisis," are due to nonresponsiveness (depletion) of the adrenal cortex.[4, 6] These two conditions, namely the increased cortical activity supposedly due to ACTH discharge, and the state of hypocorticism were thought to represent a very suitable approach to our objectives.

METHOD AND PROCEDURE

Thirty-eight albino rats of the Sprague-Dawley strain of both sexes and with an average weight of 250 grams were used. They were fed Purina Dog Chow that was enriched, in the case of adrenalectomized rats, with milk, meat, and carrots. All animals were trained prior to the experiments in order to minimize emotional factors that may arise from handling and taking blood samples or from the restraint required for measuring capillary resistance. Not until the animals became real pets were they used for the experiments. This is emphasized because it has been shown that disregard of emotional factors may lead to erroneous results.[9]

Bilateral adrenalectomy was performed on two dogs in two stages. Following the second adrenalectomy the animals were given desoxycorticosterone (1-2 mg. daily)* until the cortisone test period, at which time it was discontinued. Throughout the experiments the animals received one per cent sodium chloride ad libitum for drinking water. One normal dog was studied in the long-term cortisone experiment and two normal dogs in the prolonged fasting experiment. All the dogs were well-trained; therefore testing capillary resistance and taking blood samples did not call for restraint or give rise to emotional upset.

The testing of cortisone in the adrenalectomized rats (Experiment 2) was performed in the late postoperative state, i.e., six weeks following operation. As has been previously shown,[4] this late postoperative period is more suitable for testing hormonal effects than is the early stage. Cortisone† was administered for eight days subcutaneously in daily doses of 1 mg./100 Gm. In Experiment 2 cortisone was given to adrenalectomized rats for a brief period and then withdrawn, while in Experiment 3 it was administered to adrenalectomized rats and dogs continuously for thirty-five to seventy-eight days. In Experiment 4 cortisone was given to a normal dog for thirty-six days. In order to rule out the nonspecific effect of the cortisone vehicle, this dog was treated for seven days with corresponding amounts of this vehicle after termination of the experiment, i.e., when eosinophils and capillary resistance had returned to control values.

Stress by forced muscular work (Experiment 5) was inflicted in the form of swimming. The rats were placed in a tank of water at 37° C. for eighty minutes on three successive days. For the cold stress (Experiment 6) the animals were kept at –2° C. for forty-eight hours. In Experiment 7 a normal dog was fasted (water ad libitum) for twenty-five days.

Control base lines of capillary resistance and hematologic data were established before any intervention detailed previously and the tests were continued thereafter at one- to two-day intervals. Blood samples for hematological study were taken from the tail of the rats and from the leg veins of the dogs. The level of circulating eosinophils was determined by the method of Randolph[10] in the modification of Henneman, Wexler and Westenhaver.[11] Capillary resistance was tested by the negative pressure method on the abdominal skin surface of the animals. The details have been described elsewhere.[4]

*Desoxycorticosterone acetate aqueous macro suspension (Bell Pharm. Co.).

†11-dehydro-17 hydroxycorticosterone 21-acetate (Cortone acetate, Merck & Co., Inc.).

RESULTS

1. *Effect of Adrenalectomy on the Capillary Resistance and Eosinophil Level in the Normal Rat (Fig. 1).*—In agreement with previous findings[3, 4] the capillary resistance was found to be unaltered during the first and second day following adrenalectomy. Between the third and the fifth day (depending on the environment temperature) it began to drop, at first slowly, then precipitously, to very low levels. If the removal of the adrenals was complete and

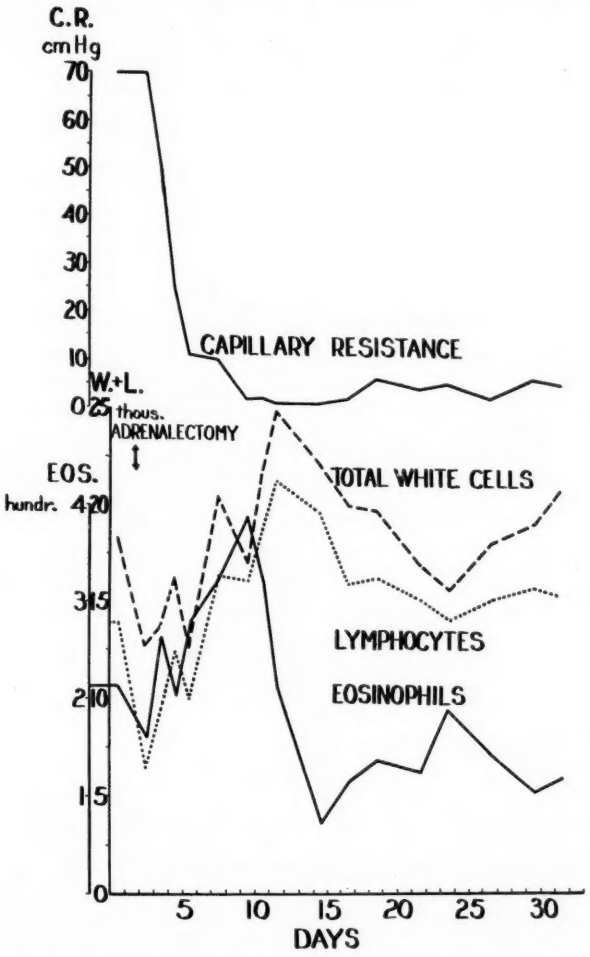

Fig. 1.—Effect of adrenalectomy on the capillary resistance and circulating eosinophils of the albino rat. Correlation is present in the early postoperative state only.

the animal had no accessory tissue, the resistance remained at a low level permanently, never exceeding 10 cm. Hg. Simultaneously with this drop in the capillary resistance the number of eosinophils increased significantly. This eosinophilia, however, was only a transient phenomenon; in most cases the eosinophil count returned to average normal values within a few days.

2. *Effect of Administration and Withdrawal of Cortisone on the Capillary Resistance and Eosinophil Level in the Adrenalectomized Rat (Fig. 2).*—

In these experiments the capillary resistance was found to be permanently at a very low level during the entire period prior to cortisone administration. No characteristic pattern of the eosinophils was seen during the same time. Only 3 animals out of 22 revealed an eosinophil count around 600 and only one was found with counts varying between 420 and 1,200. All the others had normal or even subnormal values.

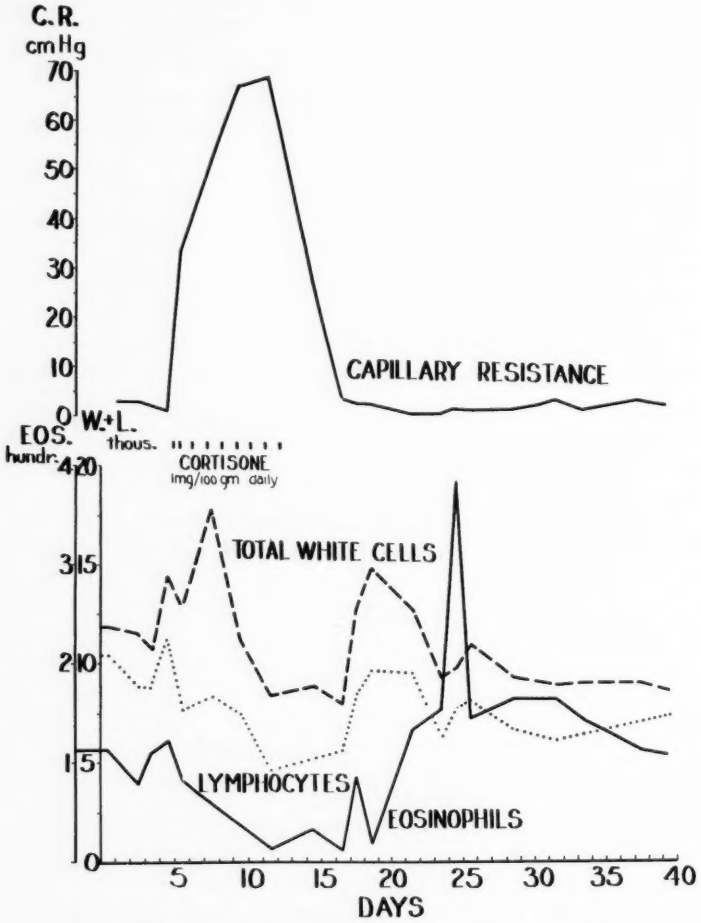

Fig. 2.—Effect of daily administration of cortisone (1 mg./100 Gm.) on the capillary resistance and eosinophils of the adrenalectomized rat in the late postoperative period. Good inverse correlation during cortisone treatment and immediately after its withdrawal. Correlation fades during continuous absence of cortisone.

The response to cortisone was typical and was observed in all animals in this group: a sharp rise in the capillary resistance, a maintained high level so long as cortisone was given, and a rapid drop when it was discontinued. The eosinophil counts presented an inverse relationship to this pattern, with the exception that when cortisone was withdrawn and marked eosinophilia set in, this proved to be a transient condition in comparison with the capillary resistance, which remained permanently low.

3. *Effect of Prolonged Administration of Cortisone on the Capillary Resistance and Eosinophils in the Adrenalectomized Rat and Dog.*—Fig. 3 is a representative graph of a group of three rats demonstrating the typical inverse movement of the capillary resistance and the eosinophils when cortisone was begun. It also indicates that capillary resistance remained high and eosinophil level depressed as long as cortisone was being administered (seventy-eight days). Similar observations were made on two adrenalectomized dogs.

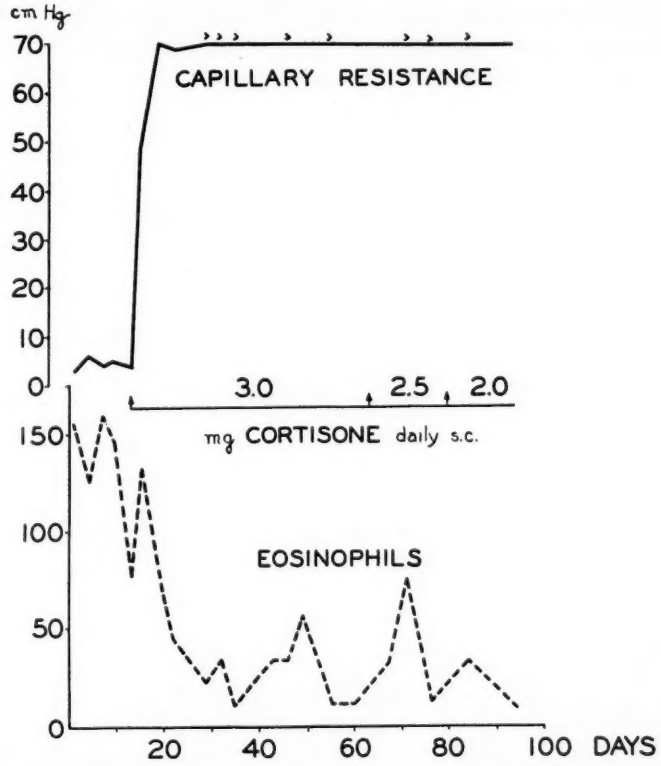

Fig. 3.—Effect of daily administration of cortisone on the capillary resistance and eosinophils of the adrenalectomized albino rat in long-term experiment. Note the perfect correlation during the entire experiment.

4. *Effect of Prolonged Administration of Cortisone on the Capillary Resistance and Eosinophil Level in the Normal Dog.*—The control base lines established during fourteen days show (Fig. 4) low normal capillary resistance values and a tendency to eosinophilia with wide fluctuations of the counts. Daily administration of cortisone (15 mg. intramuscularly) depressed the eosinophils to a rather constant low level with minimal variations and increased the capillary resistance. The new levels remained unchanged as long as cortisone was given. When, after thirty-six days, it was withdrawn a typical inverse movement set in and the former levels were again obtained. Following this the cortisone vehicle was administered, but no significant change in capillary resistance or eosinophil values was obtained.

5. *Effect of Forced Muscular Exercise Upon the Capillary Resistance and Eosinophils in the Normal Rat.*—As exemplified in Fig. 5 the first phase of response, the initial rise in the capillary resistance, was not conspicuous because the previous control values were also very high. The concomitant drop in the number of eosinophils was also of a moderate magnitude and doubtful significance. During the second and third phases (capillary crisis), however, the inverse relationship between capillaries and eosinophils became more apparent: As soon as the capillary resistance began to decrease, eosinophilia set in and persisted during the entire period of low capillary resistance. With the restoration of the latter, the number of eosinophils sharply dropped, then gradually returned to control level.

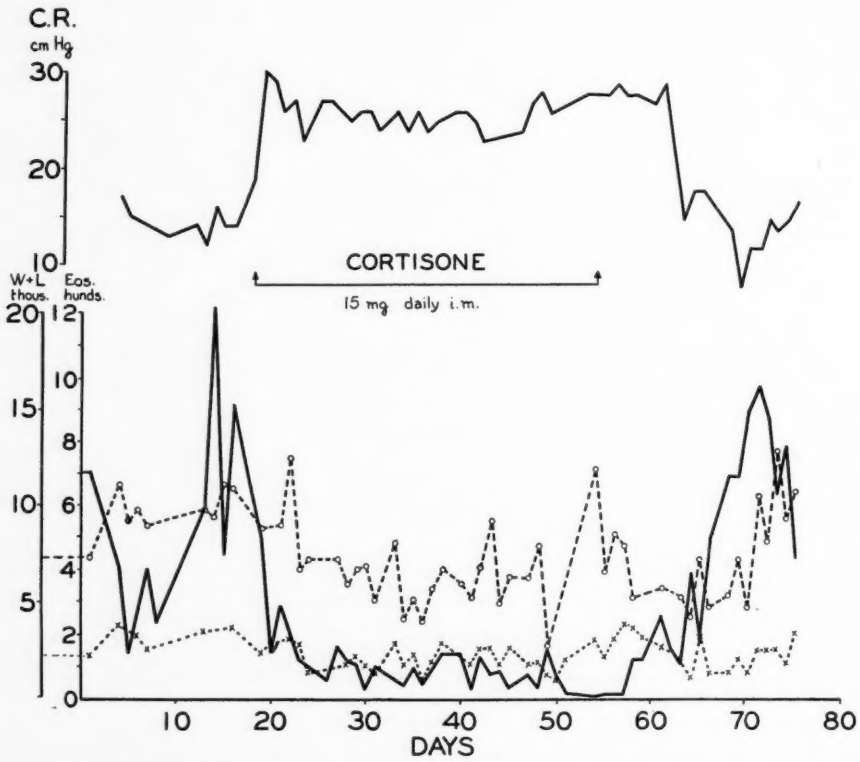

Fig. 4.—Effect of administration of cortisone (15 mg. daily) on the capillary resistance and circulating eosinophils of a normal dog in long-term experiment. Perfect correlation during the entire experiment.

6. *Effect of Cold Stress Upon the Capillary Resistance and Eosinophils in the Normal Rat.*—As seen in Fig. 6 the pattern is about the same as after muscular exercise.

7. *Effect of Prolonged Fasting Upon the Capillary Resistance and Eosinophils in the Normal Dog.*—In the two foregoing experiments on rats the capillary resistance had a high normal control period so that no significant further rise could be demonstrated upon the application of a stress. The dog

used in this experiment had a high normal control level of capillary resistance for this species but relatively low as compared to rats. This fact explains that the first phase of the stress response, the rise in the capillary resistance, was not only easily demonstrable but also very spectacular (Fig. 7). During the first days of fasting the capillary resistance gradually increased, and on the fourth day it reached the maximum recordable height (>70 cm. Hg). This high level was maintained, interrupted by some fluctuation, throughout the entire period of fasting (twenty-five days). The eosinophils showed wide variations in the period prior to fasting. After the third day of the fast their number dropped precipitously and remained at a constant low level.

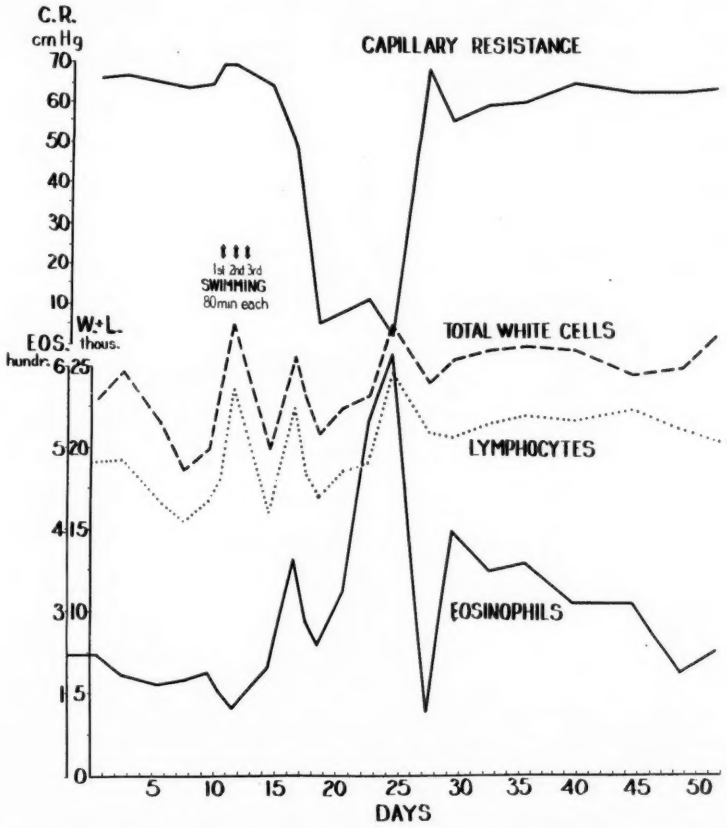

Fig. 5.—Effect of forced muscular exercise on the capillary resistance and eosinophils of the normal albino rat. Note the "capillary crisis" accompanied by eosinophilia.

The total white count and the lymphocytes did not offer any characteristic pattern except in Experiment 4. Fig. 4 demonstrates a tendency toward a persistent depression during the administration of cortisone.

DISCUSSION

In their study of the capillary resistance of patients with rheumatoid arthritis, Robson and Duthie[1, 2] observed that during treatment with ACTH or

cortisone, the rise in the capillary resistance was consistently accompanied by a fall in the eosinophils. They also found that nonspecific stress, such as x-ray irradiation, nitrogen-mustard, or fever therapy, as well as epinephrine or insulin, had a similar effect. The experiments presented here indicate that although there is a close correlation between these two phenomena, there is also an essential difference between them.

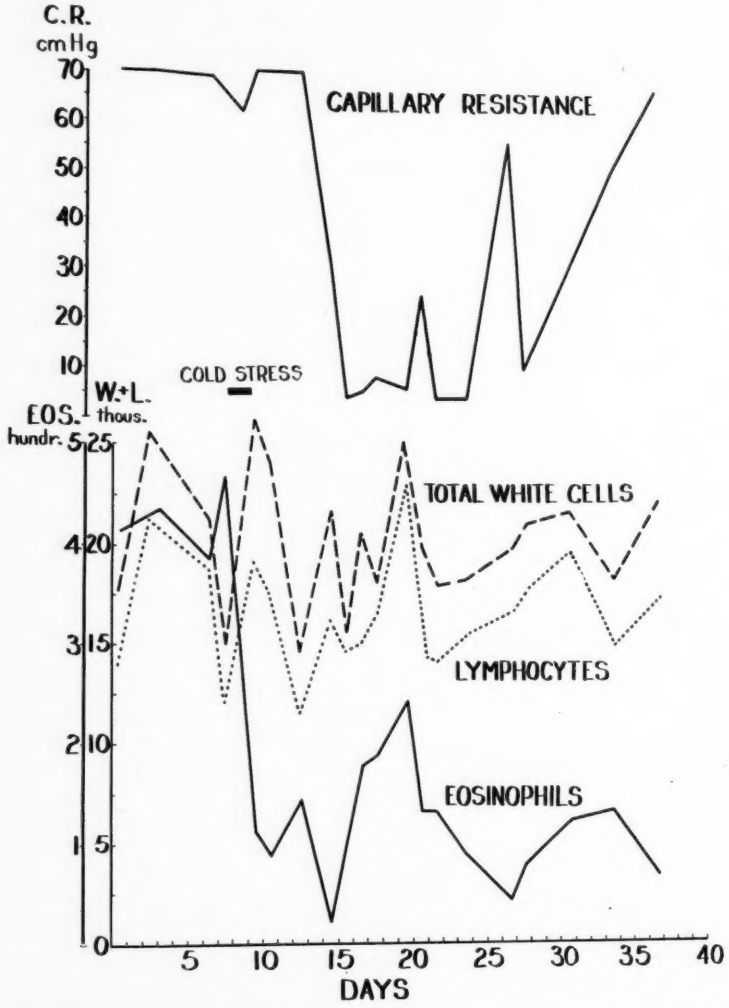

Fig. 6.—Effect of cold stress on the capillary resistance and circulating eosinophils of the normal albino rat. The drop of eosinophils in the first phase of stress response is spectacular; the eosinophilia during the ensuing capillary crisis is moderate.

Animals subjected to cold, muscular exercise, or prolonged fast revealed an impressive inverse relationship which was especially spectacular during the capillary crisis. At this time, in the second to third week following stress, the adrenal cortex appears to be nonresponsive, presumably as a result of depletion. The depressed cortisone level is well reflected in *both low capillary resistance*

and high eosinophil count. Following adrenalectomy in the normal rat there is also a good correlation *during the first week.* In the following weeks, however, the correlation begins to fade. While the capillary resistance remains permanently low, thus constantly signaling the absence of cortisone, the eosinophils drop to a noncharacteristic level. The same was demonstrated in the experiments in which cortisone was given to and withdrawn from adrenalectomized rats in the late postoperative period. The inverse movement that sets in when the depressing effect of cortisone has worn off, was again only of a few days' duration because the high eosinophil counts soon return to average values.

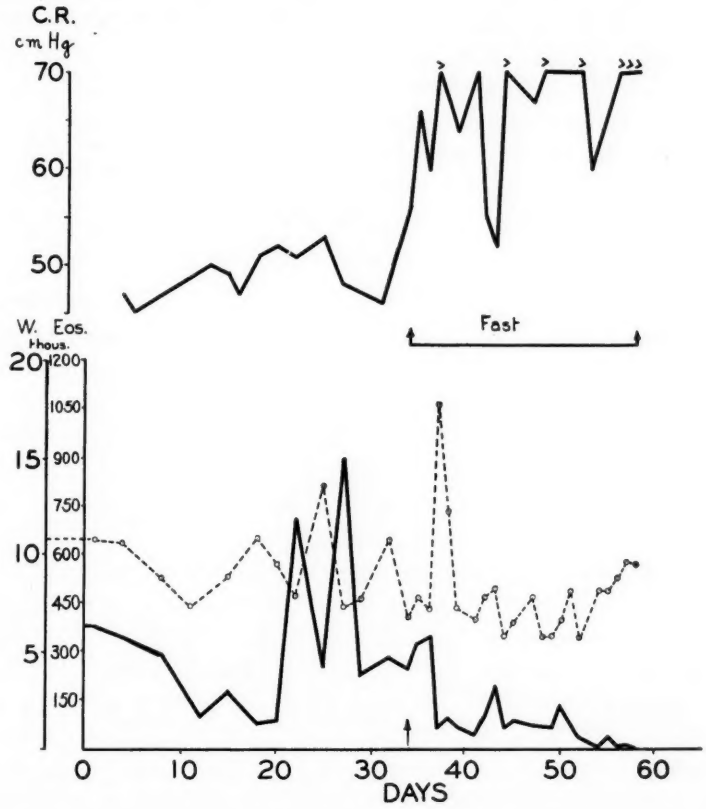

Fig. 7.—Capillary resistance and circulating eosinophils in the normal dog during prolonged fast. Good correlation during the entire experiment.

This failure of the eosinophil count to reflect the cortisone level when it stays persistently low during a long-term experiment contrasts sharply to the findings in Experiments 3, 4, and 7, indicating that the eosinophil level remains depressed as long as a higher cortisone concentration prevails. As in this case the high capillary resistance also remained constant, there was a perfect correlation between these two indicators of cortisone. It should be pointed out that during the depression of eosinophils by cortisone the daily variations were much smaller when compared with periods without cortisone.

How to explain this different behavior of the eosinophils in case of high and in case of low cortisone level in the prolonged experiment? Why is there a good correlation between capillary resistance and eosinophils at the moment the cortisone level changes? Why the good correlation if cortisone level remains high, and why the discrepancy if cortisone level remains low in the long-term experiment?

The following explanation may be offered. Capillary resistance seems to be regulated mainly by cortisone, whereas the level of circulating eosinophils is dependent on a variety of factors, cortisone being only one of them. Even if the mechanism of the depressant action of cortisone upon the eosinophil count is not yet clearly understood, there is no room for doubt that this action is a positive function of cortisone. The effect of any decrease in cortisone concentration upon the eosinophils must, therefore, be considered as a negative function. It seems that the depressant action of cortisone is so predominant that in its presence no other regulatory factors can become effective or manifest. When the depressory action of cortisone decreases, the resulting eosinophilia is likely to be compensated and the high count brought down to normal sooner or later by some of these factors.

SUMMARY

The correlation between capillary resistance and circulating eosinophils was studied in seven experimental conditions: following adrenalectomy in the normal rat and dog, during administration and withdrawal of cortisone in the adrenalectomized rat, during prolonged administration of cortisone in the adrenalectomized rat and dog, during prolonged administration of cortisone in the normal dog, after cold stress and forced muscular exercise in the normal rat, and during prolonged fasting in the normal dog.

A good inverse correlation was found in any situation presumably connected with a *change* in cortisone concentration of the body. (a) If cortisone concentration *increases* (cortisone administration to adrenalectomized or normal animals, first phase of stress response presumably associated with ACTH discharge) the capillary resistance rises and the eosinophils drop. (b) If cortisone level *decreases* (cortisone withdrawal in adrenalectomized or normal animals, adrenalectomy in normal rat, second phase of stress response presumably connected with cortical hypofunction) the capillary resistance precipitously drops and eosinophilia occurs.

A good inverse correlation (persistent high capillary resistance and persistent low eosinophil level) was found in any situation in which a *high* cortisone level was maintained during a long period of time by either exogenous (cortisone administration) or endogenous method (protracted stress).

No persistent correlation was found in any situation connected with a permanently *low* cortisone level in the animals. After adrenalectomy or cortisone withdrawal there is at first a seesaw movement: the capillary resistance drops and eosinophilia sets in. This good correlation fades, however, when the cortisone level remains low in that the high eosinophil count returns to normal

average levels whereas the capillary resistance remains permanently low. In these conditions the eosinophils cease to reflect the cortisone level and only the capillary resistance remains to be a reliable indicator.

We are indebted to Drs. C. M. Wilhelmj and H. H. McCarthy for their interest and valuable assistance. Acknowledgment is made to Mrs. N. Nelson for her excellent technical assistance.

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THE APPLICATION OF VITAMIN B_{12} TOLERANCE TESTS TO AMERICAN AND MEXICAN SUBJECTS

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VITAMIN tolerance tests have been used both to estimate the degree of saturation[1] and to provide one type of base-line data from which dietary requirements[2] may be estimated. With sufficient background information in hand, such tests offer a means of establishing the relative adequacy of dietary sources of the vitamins under study. Tolerance tests using vitamin B_{12} have been reported in earlier studies.[3, 4] This communication extends those studies to a group of Mexican men with a dietary history of a low vitamin B_{12} intake in comparison with a group of Americans in substantially the same age range but with more abundant intake of vitamin B_{12}-containing food.

EXPERIMENTAL PROCEDURE

Choice of Subjects.—In the entire series, a total of 98 men were studied. The Mexican subjects,* all residents of Mexico City, were selected because their dietary histories indicated a chronically low intake of vitamin B_{12}. Mean age in this group of 51 was 24 years; range 18 to 39 years. Dietary vitamin B_{12},[5] as far as could be ascertained, was limited to that contained in one small portion of meat and cheese per week, and in some instances, milk. The total B_{12} intake was estimated to be less than 0.2 μg per week. Dietary proteins were primarily of vegetable origin (soybean, corn, etc.); rarely eggs were included. Mean intake was estimated to be 3,000 calories per day. Upon examination all volunteers were found to be clinically healthy at the time of injection. Inquiry into any serious illness during the twelve months prior to the study revealed that only three had histories of acute infections (lobar pneumonia and typhoid fever).

The American volunteers, 47 men, were drawn from students in the School of Hygiene and Public Health and staff members of a Baltimore hospital. Survey of their dietary history revealed that their intake of vitamin B_{12} was derived from liberal amounts of milk, meat, cheese, etc. and estimated to range from 1 to 5 μg per day. The mean age of the American group was 29 years (24 to 47).

The Tolerance Test.—The tolerance test was essentially carried out according to the procedure described previously.[3] In brief, the subjects voided before injection; these samples served as basal specimens. A single test dose of crystalline vitamin B_{12} solution (65, 50, 40, 30, or 20 micrograms) was then given intramuscularly to each individual. No restriction was imposed on daily activity. In most cases urine specimens were collected for only eight hours, since extension to twenty-four hours or even forty-eight hours did not result in an increase of more than 5 per cent in the total urinary excretion of the vitamin. Hence, only the

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The work was supported by grant-in-aid from the Squibb Institute for Medical Research.

Crystalline Rubramin (65 μg per ml.) with ½ per cent phenol was kindly supplied by Dr. G. Rake of the Squibb Institute for Medical Research.

Solutions of crystalline vitamin B_{12} (20 and 30 and 100 μg) with no preservative were generously supplied by Merck & Co., Inc.

Received for publication, Sept. 25, 1953.

*Very few incidents of nutritional macrocytic anemia have been observed in Mexican people of this economic group.

microbiologic activities in the eight-hour specimens were used in comparing the groups. After measurement of the volumes of the urine, two aliquots (10 ml.) of each sample were stored in sealed ampules under toluene at 5° C. The specimens from the Mexican volunteers were shipped by air express from Mexico City to Baltimore for microbiologic analysis. The fact that standard solutions of vitamin B_{12}, flown to Baltimore along with the eight-hour specimens, underwent no loss in activity suggests that the B_{12} in the urine samples was likewise unchanged in transit. Aliquot samples of urine from the American subjects in Baltimore were kept under toluene in the cold room until assayed.

The Determination of Microbiologic Activity.—The microbiologic activities in the urine specimens (basal as well as eight-hour ones) were determined according to the procedure of Skeggs and co-workers.[6] Recovery experiments performed by adding known amounts of crystalline vitamin B_{12} to basal urines of both Mexican and American subjects yielded quantitative results and thus indicated the absence of any inhibitor.

RESULTS

The results of three separate experiments are tabulated in Table I. Table I does not include values for the 30 μg dose. The 8 Mexican subjects receiving this dose showed a much larger mean value (7.2 ± 1.8) than the mean excretion of 21 Mexican subjects (in 2 separate experimental groups) given 50 μg injections and that of 5 subjects receiving forty micrograms. This abnormally large average of the 30 μg dose was due to three specimens whose individual values were higher than the largest ones in the 40 or 50 μg groups. For this reason the observed mean value at the 30 μg dose appears to be an artifact, and further data will have to be accumulated at this test dose level. From Table I, it is apparent that less vitamin B_{12} activity was found in the urine specimens from Mexican individuals than in those from Americans at all levels of test injection shown. The difference at each reported test dose was significant ($P<0.05$). When all three trials with regard to dosages are analyzed collectively, the difference becomes very highly significant ($P<0.001$). In this study only men were used. The mean age and body weight of the Mexican subjects were lower than those of American subjects. The slight difference in age is not believed to be significant.[4] The difference in body weight would make our observed differences in vitamin B_{12} excretion even more marked if our results were expressed as micrograms retained per kilogram. Hence, it may be concluded that the

TABLE I. URINARY EXCRETION OF VITAMIN B_{12} BY MEXICAN AND AMERICAN SUBJECTS

EXPERIMENT NO.	DOSAGE (μG)	MEAN AGE (YEARS)		MEAN BODY WEIGHT (KILOGRAMS)		VITAMIN B_{12} IN URINE* (μG)	
		M	A	M	A	M	A
1	65	21	25	56	73	17.1 ± 2.5 (9)	20.2 ± 1.6(8)
	40	20	—	56	—	4.9 ± 0.78(5)	9.0†
2	50	22	29	60	75	5.2 ± 0.69(11)	12.8 ± 1.2(11)
3	50	25	—	60	—	5.1 ± 1.3 (10)	—
	20	27	30	57	67	0.57 ± 1.3 (8)	1.6 ± 0.2(11)

M = Mexicans

A = Americans

*Microbiologic activity of vitamin B_{12} in eight-hour urine specimens in micrograms ± standard error of the mean. The numbers in parentheses give the number of individuals in a given trial.

†Value obtained by interpolating a composite curve plotting excreted B_{12} versus injected dose.

Mexican individuals to whom the tolerance test was applied retained a larger amount of the injected vitamin B_{12}; this difference was not due to age or body weight.

DISCUSSION

Exogenous (dietary) vitamin B_{12} does not have any immediate effect on urinary microbiologic activity in the basal specimens since it is poorly absorbed from the gastrointestinal tract.[7] Hence, in experiments of this sort it is not necessary to estimate or control the dietary vitamin B_{12} intake shortly before or during the collection period. The greater amount of B_{12} retained by the Mexican subjects may be a reflection of lower tissue reserve resulting from their chronically low intake of vitamin B_{12}. If such a situation actually exists, one might apply the "saturation" test by estimating the amounts of vitamin B_{12}

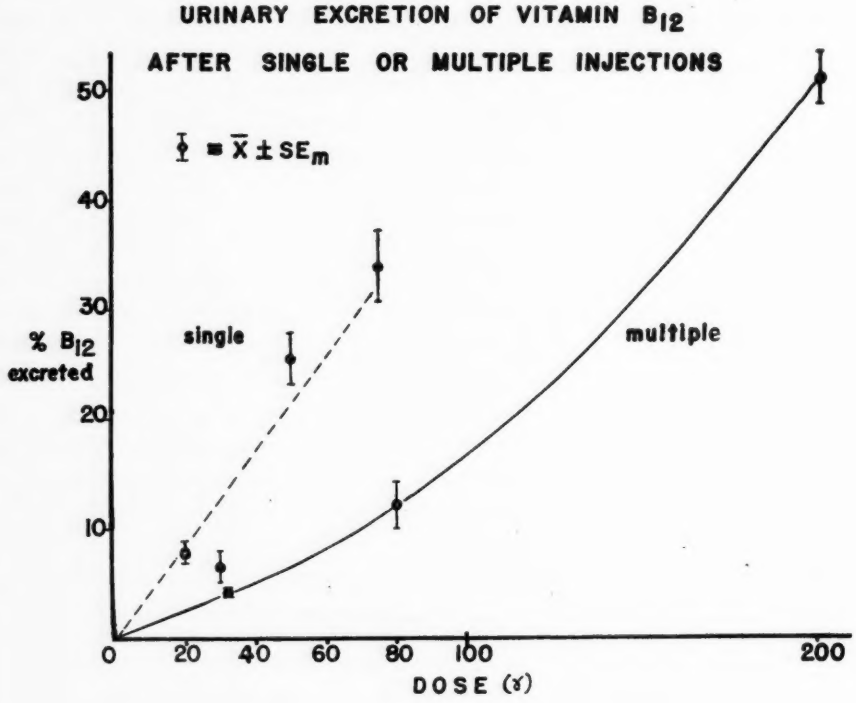

Fig. 1.

to be administered in order to "saturate" the tissues of these two groups of individuals, by single or multiple injections. Since earlier reports[3, 8] demonstrated that retention is also a function of dosage of vitamin B_{12} given by single injection within the ranges studied, such a procedure will consequently not yield any constancy. Attempts to "saturate" clinically healthy American subjects in groups of 5 to 6 each by daily successive injections of various doses of the crystalline vitamin were made, and the urine specimens were collected every twenty-four hours. For comparison, another group of comparable subjects was

given corresponding total amounts in one injection. The microbial activities in the twenty-four hour urine specimens of both groups were measured. It can be seen from Table II that the amounts of vitamin B_{12} activity in the urine increased with successive daily injections, particularly at the two highest dosages. Furthermore, 3 daily injections of 50 μg were not sufficient to "saturate" these subjects, so that as much as 40 per cent of the fourth injected dose was retained. Fig. 1 shows two lines which plot the percentage of the injected vitamin found in the urine against the dosage administered, either as single or multiple injections. They demonstrate that subjects receiving vitamin B_{12} by multiple injections retained more than those receiving the same amounts by single injection; this difference increased with dosage. These findings, with both types of test, indicate that it is impossible to determine any saturation dose either by single or multiple injections.

The results of our earlier experiments[9] on rats suggested that animals previously dosed with vitamin B_{12} excreted more vitamin in the basal urine and retained less of a subsequently administered dose of radioactive B_{12} than those animals fed on a vitamin B_{12}-deficient diet. These data are in harmony with the hypothesis that organisms deficient in a certain nutrient are apt to retain a larger amount in the tissues upon subsequent injection.

While it is attractive to explain our data on the difference in the excretion of the injected vitamin B_{12} by the Mexican and American individuals on the dietary basis, the possible influence of environmental factors such as temperature, etc., or of physiologic phenomena such as kidney function has not been eliminated. Hence, this communication should serve only as a record of the observed difference with explanations to be tested by further experiments.

SUMMARY

Different dosages of crystalline vitamin B_{12} were injected intramuscularly to 51 healthy Mexican subjects, all residents of Mexico City. The mean age in this group was 24 years, with a range of 18 to 39 years. Identical amounts of this vitamin were also administered to a comparable group of 47 American subjects drawn from residents in the city of Baltimore. The determination of the urinary excretion following injection showed that less vitamin B_{12} activity was found in the urine specimens from Mexican individuals than in those from Americans at all levels of test injections. This difference at each reported test dose was significant (P—less than 0.05). These findings are attributed to the differences in the dietary intake of vitamin B_{12}, with that of Mexicans at least 30 times less. Other possible explanations, however, such as influence of environmental factors or kidney functions have not been eliminated.

We wish to acknowledge with thanks the technical aid of Mr. D. Gleysteen and Mr. Bryant Chow.

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MISSED CONTAMINATIONS IN BIOLOGIC PRODUCTS: THE ROLE OF PSYCHROPHILIC BACTERIA

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THIS report is concerned with the occurrence of psychrophilic† bacteria (i.e., bacteria growing only below incubator temperature) as contaminants in biologic products. It is by no means the first such report; several accounts have appeared in the medical literature, in the past two decades, reporting the finding, in serums, blood, or other biologic products, of organisms failing to grow at the usual incubator temperature of 35°-37° C. and hence not detected by the usual sterility test procedures.[1-4] While this paper was in preparation Pittman[5] reported a number of such observations, with particular reference to contamination of blood and its derivatives.

Despite the intermittent appearance of such reports, the standard sterility test procedures still commonly include cultivation only at incubator temperature.[6, 7] Furthermore, the frequency of such contaminants (one-quarter of all proved contaminants in our series), the range of psychrophilic microbial species implicated, and the variety of biologic products so contaminated has in our experience been broader than studies published to date would suggest. Our findings are, therefore, presented in some detail‡ in order to emphasize certain difficulties which have been found to arise in sterility testing, and to provide evidence of the necessity for additional precautions in sterility testing of biologic products.

METHODS

The Biologic Laboratory has followed the sterility testing procedures recommended by the National Institutes of Health (and its predecessor, the Hygienic Laboratory) in their official memoranda and informal communications, since the Biologic Laboratory (then the Antitoxin and Vaccine Laboratory) received a license for manufacture and sale of biologic products in 1918. Prior to 1941, the test was performed in beef or veal infusion broth in freshly de-aerated Smith tubes, under the general directions provided by the Memorandum of Details of 1934.[8] With the introduction of the fluid thioglycollate medium,[9] its adoption [10] and later modification[11] by the N.I.H., and the revision of the Memorandum of Details,[12] the laboratory followed the basic principles and methods set forth in these documents, with certain added precautions such as extended size or number of samples when these appeared indicated. Temperature of the incubator, at the levels normally used, was 35°-37° C.

From the Biologic Laboratory, Institute of Laboratories, Jamaica Plain, Boston, Mass.
Presented in part, at the 47th General Meeting of the Society of American Bacteriologists, Philadelphia, Pa., May 13, 1947, and at the April 26, 1949, meeting of the Northeast Branch, S.A.B.
Received for publication, Sept. 24, 1953.

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†The term "psychrophilic" is variously defined in medical dictionaries, since "psychros" (cold) means different things to different people. The term appears to describe, better than any other familar word, the principal characteristic of the organisms with which this paper is concerned, namely, growth only at temperatures below incubator temperature. A more precise word, e.g., "mesothermophobic," could be concocted but could scarcely be spoken trippingly on the tongue. Therefore, the word "psychrophilic" will be used throughout this report to refer to bacteria growing only below 35°-37° C.

‡An abstract of the earlier phases of this study has been published (J. Bact. 54: 31-32, 1947).

Following the first observation in 1939 that a contaminant missed by incubation at this temperature would grow at room temperature (usually 20°-25° C.*), all sterility tests were carried out both at incubator and room temperatures. For reasons of economy, strictly quantitative duplication of the standard test was not attempted, the size of the sample employed for the room temperature test being smaller than in the routine test.

Culture tubes on test have been inspected routinely on the second, fourth, and seventh days, and smeared and cultured wherever any doubt existed as to their freedom from contamination.

As is the customary practice, sterility tests were taken, not simply on the occasion of bulk filtration or filling into final containers, but also at various stages of processing or storage of many products, to provide a check on the cleanliness, sterility, or general reliability of various methods and procedures in use.

RESULTS

Characteristics and Distribution of Room Temperature Contaminants.— During the eleven-year period 1941 through 1951, the occurrence of contaminants detected in the Biologic Laboratory was as shown in Table I.

TABLE I. INCIDENCE OF CONTAMINANTS OF BIOLOGIC PRODUCTS, 1941 THROUGH 1951

YEAR	TOTAL PROVED CONTAMINANTS	ROOM TEMPERATURE CONTAMINANTS	
		GRAM-NEGATIVE RODS	OTHER
1941	20	2	1 gram + coccus
1942	18	8	0
1943	22	3	0
1944	32	7	1 gram + coccus
1945	36	1	0
1946	26	13	1 gram + coccus 1 yeast 2 gram + rods
1947	26	1	1 yeast 1 mold 1 gram + coccus
1948	29	7	5 yeasts
1949	23	2	2 yeasts 1 mold 1 mixed gram + rods and cocci
1950	17	1	1 yeast
1951	12	1	1 mold
Total	261	46	20

Thus approximately one-fourth of all contaminants picked up during this period were psychrophilic. About two-thirds of these were gram-negative rods, motile, usually fairly large, and uniform in shape, although some strains have been small and moderately pleomorphic. Growth on plain agar or in plain broth in most instances produces yellow, green, or bluish pigment; hemolysis is frequently present on blood-agar cultures. No common sugars except dextrose are fermented; indole is not formed, and gelatin is liquefied by some but not all strains. Growth in thioglycollate medium characteristically begins at the junction of the oxidized and reduced layers, later spreading densely upward and to a lesser extent downward. Detection of the type of organisms described has sometimes been complicated by the tendency for

*This represents the usual temperature range observed in the room used; on hot summer days the temperature occasionally went 2° to 3° C. higher.

certain batches of thioglycollate medium to form a hazy turbid zone at the junction of the oxidized and reduced layers. In view of the preference of this general type of contaminant for primary growth at this point, particular care is required to determine whether such turbidity is due to bacterial growth or to a chemical precipitate.

Similar strains have been isolated from tap water at the laboratory, and from plates exposed to the air. Attempts to classify each strain by species have frequently been unsuccessful, but the data observed suggest that these organisms belong as a whole to the genus Pseudomonas. Difficulties with these or similar organisms as psychrophilic contaminants in biologic products have been reported[1-5] by several other investigators, although other bacterial genera have also been found or implicated.[4, 5, 7]

The nature of the twenty miscellaneous psychrophilic contaminants is shown in Table I. There was no outstanding similarity observed among the gram-positive rods or among the gram-positive cocci. On the other hand, we frequently isolated a yeast which, grown on plain agar, was distinctly salmon-colored. This yeast was confined to blood grouping serums sent to the laboratory for final bottling from another laboratory.

Contaminants growing only at room temperature have been found in a variety of different biologic products. The 66 psychrophilic organisms detected in 1941 to 1951 were distributed in the various products listed in Table II.

TABLE II. DISTRIBUTION OF PSYCHROPHILIC CONTAMINANTS, 1941 THROUGH 1951

PRODUCT	GRAM-NEGATIVE RODS	OTHER
Animal Serums		
Antipneumococcic serum	1	0
Antimeningococcic serum	12	0
Anti *H. influenzae* serum	2	0
Diphtheria plasma	1	0
Diphtheria antitoxin	3	1 mold
Normal horse serum	6	1 mold 1 yeast
Normal horse serum (diluted)	1	0
Human Blood and Blood Fractions		
Human plasma	2	1 yeast 1 mold
Human red cell residue	1	0
Blood grouping serum	0	7 yeasts
Immune globulin (placental extract)	1	0
Immune serum globulin	5	0
Human Fraction I	0	1 mixed gram + rods and cocci
Human serum Fraction IV	1	0
Human serum albumin	1	1 yeast 1 gram + rod
Antigens		
Diphtheria toxin	5	1 gram + coccus 1 gram + rod
Diphtheria toxoid	2	2 gram + cocci
Crystalloid Solutions		
ACD solution	2	0
Procaine solution	0	1 gram + coccus

It is difficult to derive any firm generalizations from the previously mentioned data; however, it appears that contaminants requiring lower temperatures for growth were most likely to be found either in (a) products originating in the animal buildings or (b) products submitted to chemical handling such as clarification or fractionation. During such processes there are frequent stages which fall short of genuine bacterial asepsis.

The impression has also been gained that psychrophilic contaminants were more likely to appear in products containing mercurial preservatives or no preservative, than in products preserved with a phenol derivative. It has not been feasible, however, to confirm this impression quantitatively, due to the complex nature of the records involved in establishing a denominator for such a finding.

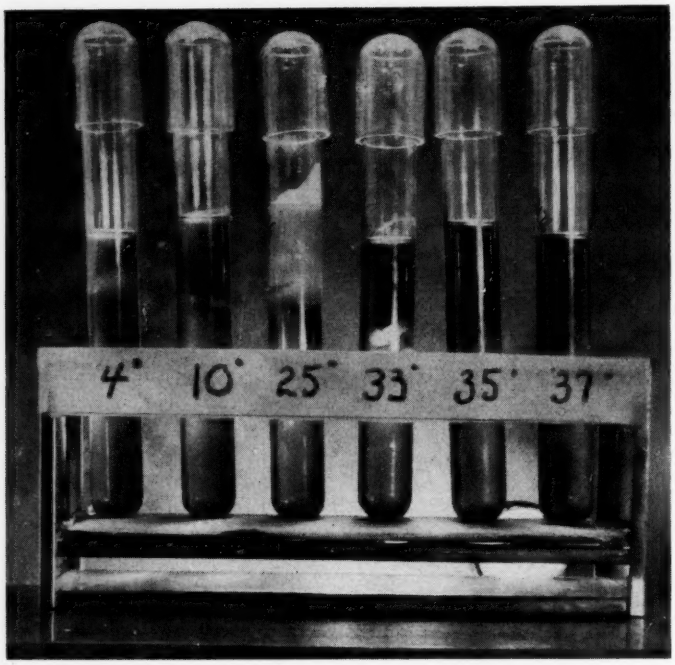

Fig. 1.—Temperature range of a typical psychrophilic contaminant. Growth was observed from 4° C. to 33° C. inclusive, being most vigorous at 25° C. No growth occurred at 35° C. or 37° C.

Temperature Range.—The range of temperature at which visible growth occurs has been studied with several strains of these contaminants. Slow growth will take place at 4° C., optimal growth is found in the usual room temperature range (20°-25° C.) and some growth can usually be observed at 33° C. None of these strains, however, has been found capable, in this laboratory, of multiplying at 35° C. or higher.* The growth of a typical strain at various temperatures is illustrated in Fig. 1.

*The first such organism isolated in 1939 was successfully cultivated at the usual incubator temperature at the National Institutes of Health, although growing more luxuriantly at room temperature.[13] We have since observed several similar contaminants which developed at both 20°-25° C. and 35°-37° C., but better at room temperature.

Cultivation at Intermediate Temperatures.—The performance of a sterility test in duplicate at two different temperatures has, as noted, been the policy of this laboratory since the first isolation of a psychrophilic contaminant, and has been recommended by the National Institutes of Health for products which "are apt to contain additional contaminants which will grow only at lower temperatures."[12] Such a procedure, however, is admittedly cumbersome. For this reason various workers have searched for an intermediate temperature which would satisfy the growth requirements of the contaminants found in biologic and similar products. Both Pittman[14] and Clapp[15] have studied the results of incubation at 31° C., and both have reported finding no contaminant incapable of growth at this temperature.*

We, therefore, undertook a parallel study of the use of 31° C. incubation for sterility testing. For the last five years of this study, contaminations occurring in routine sterility tests have been studied for temperature requirements. If the contaminant grew out on original isolation at both 35°-37° C. and 20°-25° C., it was assumed to be capable of growth at any temperature in the ranges employed, and was not studied further. Each contaminant which occurred at one temperature level exclusive of the other was studied in detail. Initially, it was grown on plain infusion agar for eighteen hours at the temperature at which it was originally isolated. The growth was washed off in a simple peptone diluent and adjusted in a Coleman Junior Spectrophotometer to an arbitrary reading. From this stock suspension, serial tenfold dilutions were made, from 10^{-1} through 10^{-9}. One milliliter of each culture dilution was planted in each of four series of tubes of thioglycollate broth. One series of culture dilutions was incubated at 37° C., one at 35° C., one at 31° C., and the fourth series was incubated at 22° C. The cultures were observed daily for one week. The end point of growth was taken as the tube with the smallest inoculum showing growth detectable on smear.

Twenty-four cultures were studied. Sixteen were isolated as contaminants from sterility tests and eight were cultures used in the laboratory and usually thought to require 37° C. incubation. Of these last eight cultures, six grew equally well at all temperatures studied, whereas two (*Corynebacterium diphtheriae, Hemophilus pertussis*) would not grow well at room temperature. Of the sixteen cultures isolated as contaminants, ten grew well at all temperatures, but six showed no detectable growth at 31°, 35°, or 37° C., while growing well at room temperature. Therefore, it appeared that the intermediate temperature of 31° C. would have failed to detect many of the contaminants in this study. Furthermore, quantitative observations on the less fastidious organisms indicated marked differences in facility of growth over the temperature range under consideration. These are illustrated in Tables III and IV, presenting the day-by-day observations on growth appearing in cultures of serial dilutions of the two test organisms used. It is apparent, in Table III, that a temperature of 33° C. favored growth only to a dilution of 10^{-3}, whereas at 25° C. the same culture grew in a dilution of 10^{-10}. Similarly, Table IV shows growth at 31° C., extending to a dilution of 10^{-4} at

*One of the 37 cultures recently reported by Pittman[5] failed to grow at 32° C.

four days and 10^{-7} at fourteen days. By contrast, a culture of 25° C. grew out to 10^{-6} and 10^{-8} at the same respective intervals. It would, therefore, appear that a small inoculum of certain psychrophilic organisms is less likely to show growth at 31° to 33° C. than at room temperature.

It has been suggested[16] that incubation at 37° C. for one week followed by another week's incubation at 20°-25° C. might increase the sensitivity of the sterility test. Several cultures were tried by this procedure. It was found that growth did not extend to the same high dilution in cultures treated in this fashion, as in cultures incubated only at 20°-25° C. In fact, it was occasionally noted (as shown in Section A of Table IV) that incubation at 37° C. completely inactivated the culture so that no growth was found. Pittman[5] reports the same observation.

TABLE III. GROWTH OF GRAM-NEGATIVE OBLIGATE PSYCHROPHILIC BACILLUS* IN THIOGLYCOLLATE MEDIUM AT VARIOUS TEMPERATURES

INCUBATION TEMPERATURE °C.	DAY OF OBSERVATION	DILUTION OF CULTURE									
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
37	4	–	–	–	–	–	–	–	–	–	–
	8	–	–	–	–	–	–	–	–	–	–
	14	–	–	–	–	–	–	–	–	–	–
35	4	–	–	–	–	–	–	–	–	–	–
	8	–	–	–	–	–	–	–	–	–	–
	14	–	–	–	–	–	–	–	–	–	–
33	4	+	+	–	–	–	–	–	–	–	–
	8	+	+	+	–	–	–	–	–	–	–
	14	+	+	+	–	–	–	–	–	–	–
25	4	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	14	+	+	+	+	+	+	+	+	+	+

*The organism used in this experiment was cultivated from bulk diphtheria toxin Lot 74E, and was typical of numerous gram-negative bacilli isolated from biologic products only by cultivation at 20°-25° C.; it was not identified as to species.

Each 30 ml. tube of thioglycollate broth was inoculated with 1 ml. of the indicated dilution of culture. Dilutions were made in peptone solution. Growth, when present, was at the anaerobic ceiling of the medium.

Total Incubation Period Required.—By chance, a rack of sterility tubes, after being read as negative at seven days on a Friday, was not discarded until Monday next. At this time it was seen to be contaminated. As a result of this observation, all sterility tests for the next six months were held for a second week. During this period, in 624 tests performed, 32 contaminants were detected only in the second week. The great majority of these were obligate psychrophils, and all but three of them were found in products containing no preservative.* It would appear likely, therefore, that very small numbers of certain organisms, particularly the psychrophils under consideration, may be inhibited from growth in the presence of a preservative, whereas in its absence they will *slowly* multiply. This may account for the occasional appearance—in this laboratory and elsewhere—of a contaminant in the filled product following a negative sterility test on the bulk batch:

*A series of contaminants in agar, detectable after fourteen days' but not after seven days' incubation, and caused by a gram-negative, facultatively *thermophilic* spore-bearing bacillus, has been reported by Townsend and Zach (J. Bact. 46: 269-271, 1943).

Immune serum globulin Lot IIG48NP, 1,000 ml., containing no preservative, was put on bulk sterility test on Sept. 26, 1942, 10 ml. at 37° C., and 3 ml. at room temperature. No growth was detected. Tests on the final containers were performed on Oct. 7, 30 ml. at 37° C. and 10 ml. at room temperature. One culture tube at room temperature showed growth. A repeat test of 10 ml. showed growth in eight tubes out of twelve at room temperature.

The experience cited has resulted in the adoption of three precautions, in performing sterility tests on products containing no preservative, viz.:

a. Take several times the usual amount of material for test (arbitrarily, three times has been selected in this laboratory).

TABLE IV. GROWTH OF GRAM-NEGATIVE OBLIGATE PSYCHROPHILIC BACILLUS IN THIOGLYCOLLATE MEDIUM AT VARIOUS TEMPERATURES

TEST	INCUBATION TEMPERATURE °C.	DAY OF OBSERVATION	DILUTION OF CULTURE									
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
A*	37	2	–	–	–	–	–	–	–	–	–	–
	37	4	–	–	–	–	–	–	–	–	–	–
	33	14	–	–	–	–	–	–	–	–	–	–
B†	33	2	–	–	–	–	–	–	–	–	–	–
		4	+	+	+	–	–	–	–	–	–	–
		14	+	+	+	+	+	+	+	–	–	–
C†	31	2	–	–	–	–	–	–	–	–	–	–
		4	+	+	+	+	–	–	–	–	–	–
		14	+	+	+	+	+	+	+	–	–	–
D†	25	2	–	–	–	–	–	–	–	–	–	–
		4	+	+	+	+	+	+	–	–	–	–
		14	+	+	+	+	+	+	+	+	–	–

*Test A: Cultures moved from 37° to 33° C. incubation four days after inoculation.
†Tests B-D: Cultures were kept at indicated temperatures. Organism used in this experiment was cultivated from bulk horse serum Lot 834-50, and was typical gram-negative obligate psychrophil bacillus. Inoculations were performed as in Table II.

b. Leave the sample to be tested (or better, the entire bulk or filled lot) at room temperature overnight before inoculating in the test medium. This principle, of incubating the sample for a period before testing it, was employed several years ago by Johnson and Meleney[16] in sterility testing of blood plasma, and has been recommended for liquid plasma by the National Institutes of Health.[17] The holding interval depends on the judgment of the laboratory; Johnson and Meleney held their samples for one week at room temperature, whereas the N.I.H. recommended two weeks.*

c. Hold the sterility tests for two weeks instead of seven days.

By the application of these precautions, the opportunity of detecting very small quantities of viable contaminating organisms was greatly increased, as shown by the data cited above.

Pyrogenicity of Room Temperature Contaminants.—Many bacterial species, but perhaps most notably the Pseudomonas and coli-aerogenes groups, have been found to contain or produce potent pyrogenic substances. Probey and

*It should be noted that if such a procedure is applied to a product containing a preservative, the sample should be held at a temperature comparable to that at which the product will ultimately be stored; otherwise certain types of contaminants, which are capable of surviving in the presence of the preservative in the cold, might be inactivated by the preservative at room temperature, and consequently missed.

Pittman[18] showed that when representative contaminants were inoculated into serums, the serums became markedly pyrogenic as measured by the usual animal tests. It appeared likely that such would be the case with certain of the contaminants isolated at room temperature in this laboratory. A typical psychrophilic gram-negative rod, isolated from a pyrogenic lot of concentrated antimeningococcic serum, was inoculated into an aliquot of a sterile, nonpyrogenic batch of normal horse serum, Lot 853-2. The serum was held for thirteen days at room temperature, then sterilized by filtration. A standard pyrogen test was then run on the product with results as shown in Table V.

TABLE V

RABBIT	WEIGHT	PREINJECTION TEMPERATURES			SERUM DOSE	POSTINJECTION TEMPERATURES				RISE
		11 A.M.	11:30	12:00	(ML.)	1:20	1:40	2:20	3:20	(°F.)
3747	7 lb.	102.3	102.2	102.3	9.5	104.3	103.8	104.1	104.8	2.5
3751	6¾ lb.	102.5	102.4	102.2	9.0	104.0	103.5	103.2	104.4	2.2
3702	6¼ lb.	102.1	102.4	102.0	8.4	104.3	104.1	104.0	104.7	2.7
Control—injected with nonpyrogenic serum										
3758	5¾ lb.	102.8	102.8	102.8	7.7	103.3	103.0	102.7	102.7	0.5

Horse serum, Lot 853-2, evidently became pyrogenic by U.S.P. or N.I.H. standards, following the introduction of the psychrophilic contaminant used in this experiment.

DISCUSSION

Sterility testing, at best, provides only limited information; a negative sterility test, properly performed, indicates only that in the sample tested there were insufficient organisms present to produce detectable growth in the medium employed during the period in question, in the presence of whatever preservative was introduced, and at the temperature specified. Aside from the statistical limitations implicit in the small-sample principle upon which sterility testing is based, and which have already been considered by others,[19] there are obvious technical limitations to the procedure. It is well-nigh impossible to employ a sufficient variety of sterility test procedures to insure adequate opportunity for growth of every conceivable contaminant. It, therefore, becomes necessary to define the most important types of contamination to be sought. These fall generally into three categories:

1. Pathogenic bacteria present in the laboratory preparing or handling the product in question, e.g., *Corynebacterium diphtheriae, Clostridium tetani, Hemophilus pertussis, Salmonella typhosa,* which generally grow best at 35°-37° C. Most of these will grow well in the usual culture media; however, some require a more complete nutritional environment than is provided by the usual standard sterility test medium; it is customary to institute auxiliary sterility tests (or, in the usual language, "viability tests") employing suitably enriched media, where such organisms may be present.

2. Pathogenic air-borne contaminants spread by a technician carrying an unrecognized infection; most agents in this class will be detected by the currently recommended sterility tests if properly employed, but some may

require special media for their detection, as previously mentioned in (1). The contamination of a biologic product, however, by a route such as this may be regarded as highly exceptional.

3. Nonpathogenic, air- or water-borne contaminants such as the Pseudomonas group and many other commonly prevalent saprophytes. It is this group of organisms with which the present report is primarily concerned.

The evidence presented above demonstrates that many members of this third group grow at 35°-37° C. either very slowly or not at all. Furthermore, as shown above, there are also a number of organisms of this general group that are not readily or rapidly cultivated at 31°-32° C. *Bergey's Manual of Determinative Bacteriology*[20] lists a large number of members of the genera Pseudomonas, Serratia, Cellulomonas, Achromobacter, Flavobacterium, Bacillus, and Clostridium, which either grow poorly or not at all above 31°-32° C. The necessity for recognition of such organisms, in the bacteriologic examination of milk and milk products, has been amply stressed[21]; their importance as contaminants of biologic products, however, has, in our experience, never been given sufficient attention. Not only is the hazard of introducing potent pyrogenic substances a real and continuing one—especially for such products as preserved whole blood or resuspended red cells—but the actual danger of systemic infection from such normally banal organisms cannot be minimized.

The findings here reported would appear to indicate that, in order to provide maximum insurance against the presence of contaminating organisms in biologic products, sterility tests should be carried out both at 35°-37° C. and at room temperature. Unquestionably, such a procedure is cumbersome. The National Institutes of Health in a memorandum dated Oct. 20, 1950,[22] have recommended "that sterility tests be conducted at a temperature of 32°-35° C., (preferably 32° C.) in all those instances where an incubator temperature of 37° C. has heretofore been specified." There is no doubt that this amendment to the previously established practices in sterility testing is a great advance, and will result in the detection of many contaminating organisms which have previously been missed. The observations cited, however, as previously mentioned, indicate that duplicate sterility testing at the more widely separated temperatures of 35°-37° C. and room temperature (±20°-25° C.) will provide more nearly complete insurance against such contamination. Furthermore, as Pittman points out,[5] incubation at one temperature alone may favor growth of one contaminant to the exclusion of others.

The findings reported in this paper and preceding studies suggest that the risk of contamination with psychrophilic organisms is greatest in those products which are either (a) derived from animal serums, (b) fractionated under conditions providing opportunity for air- or water-borne contamination, and/or (c) processed and packaged without added preservative. Additional categories of greater than normal risk of such contamination may be apparent to others who have occasion to search for such contaminants. It would, therefore, appear reasonable to apply the practice of duplicate sterility testing at two temperatures to those products in which the hazard of the oc-

currence, survival, or multiplication of a psychrophilic contaminant was greater than usual. Products stored in the cold for several days or weeks, without added preservative, would appear to be most likely to provide a favorable environment for the survival and multiplication of such organisms. Hence, as others have shown, whole blood is an outstanding offender in this respect. Obviously, one cannot expect to do a sterility test on each bottle of blood in a blood bank. Neither can one, in practice, however, test a statistically significant proportion of the samples in a filled batch of any biologic product. Therefore, with blood and blood derivatives as with other products, it will be necessary to define the probable (and indeed the possible) risks involved in the product in question, set up a safety control procedure which covers in principle the risks at stake, and adapt it as necessary to fit the special nature of the product. In this laboratory we have settled the matter by employing both room temperature and incubator temperature for every sterility test performed; in so doing, we have extended our detection of contaminants beyond any scope envisaged when the procedure was first adopted.

SUMMARY AND CONCLUSIONS

Performance of sterility tests on biologic products, under the standard conditions of incubation at 35°-37° C., has repeatedly failed to detect certain contaminants occurring in products prepared at the Institute of Laboratories and elsewhere.

The routine performance of duplicate sterility tests at 20°-25° C. (room temperature) has repeatedly demonstrated the presence of contamination not detected at the higher temperature. Sixty-six, or 25 per cent of the contaminants detected in the years 1941 through 1951 at the Laboratories, were found only by cultivation at 20°-25° C. In some years the percentage reached above 50 per cent.

Performance of sterility tests at 31°-33° C. will detect most of the organisms preferring or requiring lower temperatures for growth. Not all such organisms, however, will be detected at this temperature.

Psychrophilic contamination, in the study here reported, appears to have been largely associated with (a) processing of animal serums, (b) chemical fractionation or similar manipulation of biologic products, (c) processing and packaging without a preservative, or a combination of any or all of these conditions. In general, the hazard has been most apparent clinically in the administration of human whole blood, probably because of the large volume injected, and the difficulty in setting up adequate sterility control procedures for whole blood.

Psychrophilic contaminants represent not only a probable hazard of infection, but a well-established hazard from pyrogenic derivatives commonly liberated by such organisms. The detection of such contaminants is therefore an important component in the safety control of biologic products.

In order to insure the maximum possible opportunity for detection of such psychrophilic organisms, it is recommended that duplicate sterility tests

be carried out at both incubator and room temperatures, in those biologic products in which the hazard of such contamination cannot be excluded.

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THE PROTEIN-BOUND IODINE AS A GUIDE IN THYROID THERAPY

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IS THE serum protein-bound iodine determination a reliable guide to thyroid use?

This question prompted studies of thyroid feeding by Winkler[1] in 1945, who followed human beings on given doses for short periods of time—four weeks or less—and by Johnston,[2] who kept normal patients on the same large dose for many months at a time. (Danowski[3] recently reported regarding a similar investigation using smaller doses.)

The present study is to determine the relationship of thyroid dose to protein-bound iodine (P. B. I.) levels in both hypothyroid and euthyroid individuals.

Doses in the usual therapeutic ranges are employed over periods of six to twenty-four weeks for each dose. Observations were made for twenty months.

METHODS

Subjects were three patients with hypothyroidism of a severe degree following thyroidectomy, three euthyroid individuals, and three patients with the so-called "mild hypothyroidism" after thyroidectomy. One of these latter was thrown out of the series because of her increasing nervousness and insomnia when her thyroid dose was increased from ¼ to ½ grain daily. All others were fed thyroid extract* in doses from ¼ to 4 grains progressively. Each dose included in the averages was given over a period of at least six weeks† time and usually much longer. Protein-bound iodine‡ readings and observations on the patients were carried out every two weeks from July, 1951, until February, 1953. Serum determinations were one week apart on C. L. and on the three controls during the 4 grain dosage. Although our series was very small, our observations were numerous (307 in all).

RESULTS

The Serum-Bound Iodine in Euthyroid Subjects.—There was no increase in serum protein-bound iodine in euthyroids in any dosage given from ¼ grain through 4 grains (Table I and Fig. 1).

Severely Hypothyroid Individuals.—These patients showed a stepwise increase of the serum protein-bound iodine with each increasing dose (Fig. 1).

From the Clinical Laboratory of San Bernardino, San Bernardino, Calif.
Received for publication, Oct. 5, 1953.

*Proloid, lot No. 2810, equivalent in iodine content to U. S. P. thyroid, was supplied for our subjects by Warner-Chilcott Laboratories, Morris Plains, N. J.

†Only two observations (3 weeks) on 4 grains were carried out in the case of J.; C. L. was observed five weeks only on 1 and 3 grains.

‡All P. B. I. determinations were done by the technique of Connor, Curtis, and co-workers as modified by Maier, N. A. C. L. Bulletin, Vol. 11, No. 2, at the Clinical Laboratory of San Bernardino, San Bernardino, Calif.

TABLE I.

		P. B. I. CONCENTRATION IN MICROGRAMS % U. S. P. THYROID GRAINS PER DAY							AVERAGE INCREASE IN SERUM IODINE PER GRAIN OF THYROID GAMMA %	NO. DETERMINATIONS	CLINICAL CATEGORY
		0	¼	½	1	2	3	4			
A			(5)	(4)	(5)	(9)	(3)	(2)		28	
	J.	.8	1.4	1.7	2.0	2.5	3.5	4.7	1.0		Hypothyroid
			(8)	(5)	(11)	(3)	(5)	(2)		34	Severe
	D.	.7	1.3	1.4	1.9	2.6	3.6	5.0	1.1		July, 1951
			(6)	(5)	(7)	(6)	(6)	(4)		34	to
	S.	.7	0.9	1.3	1.8	2.3	2.7	4.5	0.9		Feb., 1953
B			(7)		(13)	(7)		(23)		50	Euthyroid
	M. C.	3.3	2.6	—	2.2	2.3	—	2.0	0.0		Controls
					(7)	(16)		(23)		48	March, 1952
	E. L.	5.7	—	—	4.8	3.3	—	3.0	0.0		to
			(7)		(24)					55	Feb., 1953
	E. D.	3.8	4.0	—	3.9	—	—		0.08		
C			(8)	(4)	(6)		(5)	(4)		27	"Mild"
	C. L.	2.2	2.2	2.3	2.1	—	2.3	2.9	0.15		Hypothyroids
			(4)	(8)	(12)	(4)	(3)	(24)		31	July, 1951
	C. O.	1.8	2.3	2.4	2.0	1.9	2.5	3.3	0.25		to
											Feb., 1953

Average Increase in P. B. I. per Grain of Thyroid: 307

A, Severe Hypothyroid — 1.0 ± 0.1 Gamma per cent — Total

B, Euthyroid — 0.0

C, "Mild" Hypothyroid — insignificant

() Superscripts indicate No. of determinations at each dose level.

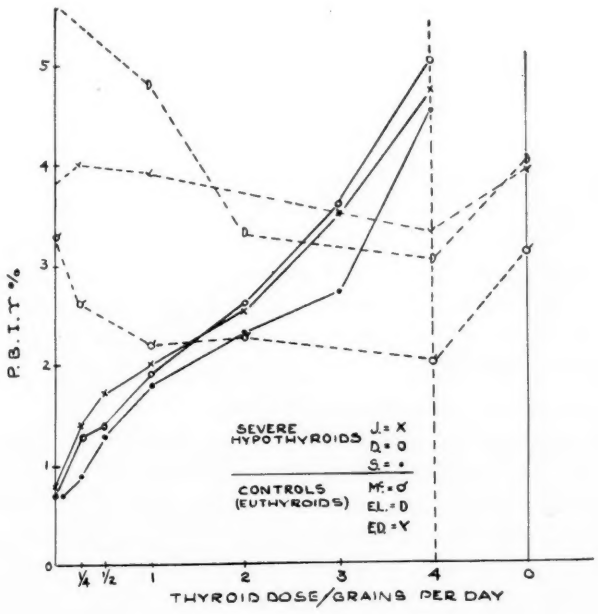

Fig. 1.—The average serum iodine levels in severe hypothyroid patients and controls (Table I).

The patients experienced an improved sense of well-being, showed a loss of myxedematous reflexes, and were clinically normal on 3 grains of thyroid. It is arresting that all three had insomnia, nervousness, tachycardia, and tremulousness on 4 grains and sought to have this dose reduced within two to three weeks after its beginning.

Mild Hypothyroid Subjects.—This interesting and always puzzling type of case seems to be an intermediate group in terms of thyroid disposal. Unfortunately, two cases are not enough, in this most questionable category, to be useful statistically.

The Relationship Between Thyroid Dose and Serum-Bound Iodine.—All patients were studied on each dose from a ¼ grain to 4 grains, with the exception of C. L., who was advanced from 1 grain to 3 grains. The control group was staggered up to 4 grains. A unilinear relationship between dosage and serum protein-bound iodine is apparent in hypothyroidism (Fig. 1). There is an average increase of protein-bound iodine of 1 gamma per grain of thyroid in the true hypothyroid.

DISCUSSION

Now that the serum protein-bound iodine has become recognized as one of the most reliable tests for the diagnosis of thyroid disorders,[4] it is desirable to evaluate its full potentiality as a therapeutic guide. Our experiment is not a new one. It is essentially the same as that of Winkler and co-workers, repeated with a smaller series of cases and more prolonged and frequent observations.

In euthyroid patients (4 in number) the previously mentioned investigators[5] had to give large doses of thyroid, 3 to 25 grains, increasing every four weeks, to effect a constant rise in the serum protein-bound iodine. They commented on the great tolerance of euthyroids for small doses of thyroid. On doses up to and through 4 grains daily our normal subjects never exhibited elevation of the serum protein-bound iodine. Throughout the treatment period a gradual depression of P. B. I. levels occurred, which was reversed with the cessation of thyroid use. Such a failure of the P. B. I. to rise in small therapeutic doses of thyroid has been observed before.[2, 3] Further, it has been shown[2] that the prolonged administration of large doses to euthyroids, that is 10 grains or more, is accompanied by a gradual fall of the protein-bound iodine from the original high levels early in administration to near normal or normal levels.

With each new dose in our stabilized hypothyroid individuals, we noticed a prompt rise in the protein-bound iodine and often a gradual return toward, but not to, the last previous level, after several weeks had passed (Fig. 2). This tendency toward lower serum protein-bound iodine levels after several weeks of thyroid administration in our hypothyroids may explain the lower ratio between dose of thyroid and final serum protein-bound iodine. This relationship equals 1 gamma per grain in our series and was 2 gamma per grain in

Winkler's, whose study was made over two dose ranges only, in several (8) individuals and for short periods of time (two to four weeks).

Perhaps our figures are open to question, if some functioning thyroid tissue is assumed to be present. The initial protein-bound iodine levels, however, in our hypothyroids are similar to those of Winkler's, the clinical findings (Table II) of myxedema were present, and the striking uniformity of the curves speaks against this.

In contradistinction to what is reported previously, the two patients in the mild hypothyroid range had curves similar to euthyroid individuals until a given dose was reached, then tolerance to thyroid medication suddenly ceased, and the serum iodine levels rose. Such intermediate activity of the

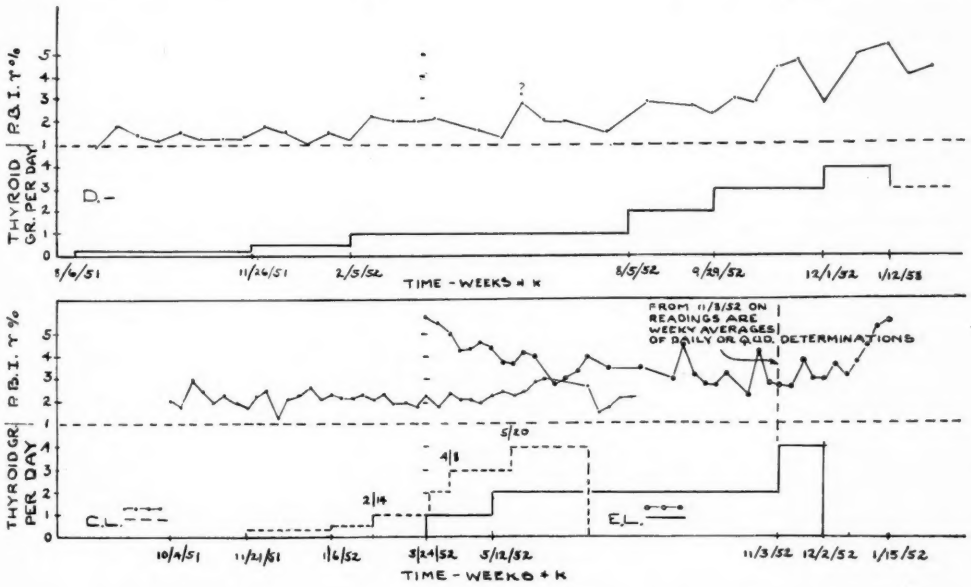

Fig. 2.—Examples of individual P. B. I. determinations over eighteen months. D., A severe hypothyroid. E., A Euthyroid. C. L., A "mild" hypothyroid.

TABLE II.

	PREOPERATIVE STATE				POSTOPERATIVE STATE			
	P.B.I.	B.M.R.	CLINICAL* STATE	PATHOLOGY REPORT	P.B.I.	WKS.	ACHILLES REFLEXES	CLINICAL* STATE
J.	3.5	0	Normal or Low	Hashimoto Struma	1.32	10	Slow Return	Low
D.	_	_	Normal or Low	"Diffuse Colloid"	0.7	3	Slow Return	Low
S.	_	_	Normal or Low	Hashimoto Struma	0.7	6	Slow Return	Low
C. L.	_	–8	Normal or Low	"Nodular Thyroid"	2.2	8	? Of rate Slow	Low
C. O.	3.9	_	Normal or Low	Hashimoto Struma	1.8	2	? Of rate Slow	Low

*"Clinical state" determined from (1) temperature tolerance, (2) pulse rate, (3) history of presence or absence of weight loss in view of appetite state and evaluated as to high, normal, or low thyroid activity.

Pathology Report: The conclusions are the pathologist's own words.

serum protein-bound iodine under thyroid administration in this group may indicate the presence of functioning thyroid tissue. This always interesting group needs further elucidation.

CONCLUSIONS

A detailed study of thyroid feeding in hypothyroid individuals and in normal controls has been conducted over twenty months' time.

The ratio of thyroid dose to protein-bound iodine levels is 1 gamma per grain of thyroid in this small group of hypothyroid subjects.

It seems possible to say that true hypothyroids may be separated from euthyroids by observation of the fixed increase in serum protein-bound iodine over intervals of six to eight weeks or longer on any given dose of thyroid medication. In accurately performed serum analyses, there should be no increase in the protein-bound iodine in euthyroid individuals and a predictable rise in hypothyroid individuals. These observatons can be made in the therapeutic range of thyroid administration. Taken in conjunction with clinical response the protein-bound iodine is a useful guide to thyroid administration.

Further work on the thyroid tolerance of "mild hypothyroids" is indicated.

We wish to express thanks to C. J. Clock, M.D., for referral of many of the patients and to W. McCandless for technical assistance.

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THE BONE MARROW AND LIVER IN IRON-DEFICIENCY ANEMIA

A Histopathologic Study of Sections With Special Reference to the Stainable Iron Content

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ALTHOUGH iron-deficiency anemia is a very common disease, relatively little attention has been paid to the histopathology of the bone marrow in this disorder. In general, only smears of aspirated marrow have been described. Scott[1] employed this technique in reporting increased erythroblastic activity in 20 cases of untreated and three cases of treated iron-deficiency anemia. He maintained that "a diagnosis of iron-deficiency anemia may be made on the sternal puncture findings with assurance." Stodtmeister,[2] on the other hand, in the study of marrow smears from 19 cases found that although the average erythroblastic activity appeared to be increased, no valid conclusions could be drawn from the marrow about any individual case. Less extensive reports of other authors[3,12] describe erythroblastic marrow smears in iron-deficiency anemia. Croizat and co-workers,[13] however, have collected 6 cases with hypoplastic marrow smears on repeated marrow aspiration.

The microscopic appearance of sections of the marrow has been described only a few times. Grawitz[14] stated that in two autopsies of chlorotic girls he could find no gross or microscopic abnormalities of the marrow, but A. Beutler[15] reported increased numbers of normoblasts in the marrow of such a patient who had been treated with blood transfusions. Dameshek[16] described markedly erythroblastic marrow in sections of sternal trephine biopsies of two patients with iron-deficiency anemia before, and one after, the institution of iron therapy. Custer[17] has published microphotographs of marrow sections from one patient with "idiopathic hypochromic anemia" and one patient with Necator americanus anemia, showing erythroblastic hyperplasia in both.

Recently, attention has been focused on the histochemical demonstration of iron in the marrow as a diagnostic tool by Rath and Finch[18] and Stevens, Coleman, and Finch.[19] The studies of these workers indicate that iron-deficiency anemia alone, of all those studied, is generally characterized by absence of stainable iron. Masshoff and Gruner[20] failed to confirm this finding, but their work has been criticized by Wenderoth,[21] Hutchison,[22] and Davidson and Jennison[23] whose results have been in agreement with those of Finch and his co-workers.

We have had the opportunity of studying marrow sections as well as smears from a relatively large number of patients with iron-deficiency anemia,

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Received for publication, Oct. 19, 1953.

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both before and at various stages after the initiation of iron therapy. In addition, a limited number of needle biopsies of the liver have been obtained. By utilizing an iron stain we have been able to observe the deposition of iron in patients receiving several forms of iron therapy, as well as to study the histopathology of this disease.

MATERIALS AND METHODS

Patients (Table I).—Twenty-three patients with uncomplicated iron-deficiency anemia are included in this study. These patients represent all such cases in which adequate marrow examinations have been made in the past two years. In each case the clinical diagnosis has been confirmed by a prompt and adequate response to treatment with inorganic iron salts orally or saccharated iron oxide intravenously. In 5 cases blood transfusions were given in addition. No liver, vitamin B_{12}, folic acid, copper, or cobalt was administered to any patient. The average hemoglobin of the untreated patients was 8.3 Gm. and the average mean corpuscular hemoglobin 23.9 micromicrograms. The iron content of the serum was uniformly greatly diminished in the untreated patients in whom it was determined. Patients with complicating diseases such as uremia, carcinomatosis, or chronic infections were excluded because of the impaired iron utilization which has been shown to exist in these conditions.[24]

Twelve marrow specimens obtained from patients without any significant organic disease served as normal controls.

Preparation of Marrow Specimens.—Forty-five sternal punctures performed with an 8 gauge needle and one surgical biopsy provided the marrow specimens used in this study. Twenty-one of these were obtained before any therapy had been instituted, 13 were from patients at various times after the treatment with iron had been begun, and 12 were from normal controls. Smears were made and were stained with Wright's stain. Fragments of marrow were fixed in Zenker's formol, dehydrated, imbedded in nitrocellulose, sectioned at 6 micra, mounted on glass slides, and placed in ether-alcohol to remove the nitrocellulose. From each specimen some slides were stained for histologic study with hematoxylin-eosin-azure (H.E.A.) (method of Maximow)[25] and others were stained for iron by placing into a fresh mixture of 1 part 2 per cent potassium ferrocyanide and 2 parts 1 per cent hydrochloric acid for one-half hour. Because of the subsequent appearance of the work of Hutchison[22] emphasizing the importance of using warm reagents and because more concentrated hydrochloric acid had been recommended by most authors,[26] additional slides from all negative specimens were stained for ten minutes in a 1:1 mixture of 4 per cent hydrochloric acid and 4 per cent potassium ferrocyanide heated to 56° C.

Interpretation of Marrow Specimens.—Each marrow preparation was read twice and identified only by number so that the clinical diagnosis was unknown. The final scoring of the smears and H.E.A. sections represents the average of the two readings.

One hundred nucleated cells were counted in the marrow smears on each reading and the average percentage of erythroblasts* was recorded. In addition, the degree of cellularity of the smear was estimated on a scale of ten.

In the hematoxylin-eosin-azure stained sections the percentage of erythroblasts was estimated to the nearest 5 per cent, since the focal distribution of these cells in sections makes differential cell counts unreliable. The percentage of the marrow occupied by hematopoietic cells as opposed to fat was also estimated to the nearest 5 per cent. The deviation of the duplicate readings from their mean averaged only 2.6 per cent erythroblasts in the counts of smears, 0.27 point in the estimated cellularity of smears, 4.1 per cent erythroblasts in the estimated per cent erythroblasts in sections and 2.1 per cent cellularity in the estimated cellularity of the sections. An attempt was also made to determine whether storage iron was present in the marrow by examining the hematoxylin-eosin-azure

*The term erythroblast is used throughout to refer to all nucleated members of the erythroid series.

sections. This was done because past experience had shown that hemosiderin is identified with ease in these preparations when it is present in increased amounts, giving rise to rather characteristic refractile green or yellow granules.

The slides which had been stained for iron were graded for the presence or absence of tissue iron stores. Some care was necessary to distinguish artifacts from true storage iron. In many preparations the Berlin blue reaction was elicited in some precipitates bearing no definite relationship to the cellular structure of the tissue and frequently lying in a different focal plane. Occasionally diffuse pale blue discoloration of large areas including blood clots was present. Such occurrences were regarded as being artifacts. The blue granules of true storage iron insinuate themselves between the nuclei of the marrow cells and are evidently an integral part of the tissue. Frequently they may be seen to lie within reticulum cells. There was rarely much difficulty in making the differentiation. As Hutchison[22] has also emphasized, a larger fragment of marrow is necessary for examination for stainable iron than for histologic study because of the irregular distribution of iron stores. In a few cases, therefore, sufficient material was obtained for adequate histologic examination, but the quantity was not sufficient for examination for iron.

Liver Biopsies.—Liver biopsies were performed with a Vim-Silverman needle using the technique described by Block[27] for spleen biopsy. H.E.A. and iron-stained sections were prepared employing the same technique used for the marrow sections.

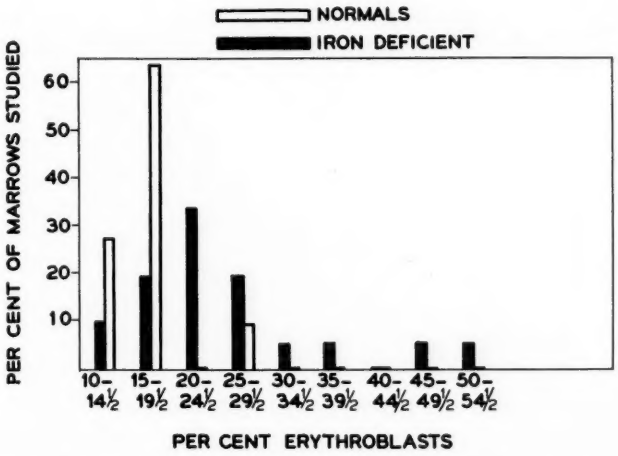

Fig. 1.—Histogram of the percentage of erythoblasts in bone marrow smears from 11 normal controls and 21 patients with untreated iron deficiency anemia.

RESULTS

Marrows.—Results of the evaluation of the marrow specimens are given in Table I, along with important data pertaining to each case.

The average percentage of erythroblasts in smears and in sections and the average cellularity of the sections is greater in the untreated anemic than in the control patients. Application of "Student's t test"[28] to the data shows that differences of the magnitude observed would be expected to occur by chance approximately 1, 10, and 1 trials per hundred, respectively. The histograms of Figs. 1, 2, and 3 make it clear that the overlap in the two groups is very large, and that therefore any attempt to establish a diagnosis on the basis of marrow examination alone would carry a large risk of error. The

TABLE I

PATIENT	SEX	HGB GM. %	RBC 10^6	MCH $\mu\mu$G	SERUM IRON γ %	TREATMENT	SMEARS		SECTIONS			
							% ERYTHRO-BLASTS (AVERAGE OF 2 READINGS)	CELLU-LARITY (AVERAGE OF 2 READINGS)	% ERYTHRO-BLASTS (AVERAGE OF 2 READINGS)	CELLU-LARITY (AVERAGE OF 2 READINGS)	PRESENCE OF IRON IN: H-E-A	PRESENCE OF IRON IN: IRON-STAINED
A. Untreated Iron-Deficiency Anemia												
1—Ka	F	4.6	2.13	21.6	—	—	12	8½	32½	65	0	0
2—Wa	F	5.5	3.40	16.2	—	—	26	3	*	*	*	*
3—Ne	F	5.8	3.32	17.5	30	—	26½	4	45	67½	0	0
4—Si	F	6.7	3.28	20.5	30	—	48½	4½	45	50	0	0
5—Li	M	7.2	2.76	26.1	—	—	18	5	57½	50	0	0
6—Tu	F	7.2	3.34	21.5	—	—	24	2	35	52½	0	0
7—Ga	M	7.7	3.10	24.8	—	—	22	7	50	60	+	0
8—He	M	7.8	4.04	19.3	—	—	35	4	40	50	0	0
9—Fr	F	7.9	3.43	23.0	50	—	22	4	47½	40	0	0
10—Vr	F	8.0	3.42	23.4	7	—	31	4	60	67½	0	0
11—Le	F	8.3	2.94	28.2	—	—	17½	6½	27½	42½	+	0
12—Ja	M	8.8	4.53	19.4	—	—	52	3	52½	50	*	*
13—Mo	F	8.8	3.00	29.3	—	—	21½	8½	47½	55	0	0
14—St	F	8.9	3.70	24.0	10	—	25½	3	40	47½	0	0
15—Wi	F	9.6	4.27	24.8	15	—	24	4½	42½	52½	0	0
16—Wi	F	9.7	4.48	21.6	20	—	16½	3½	32½	60	0	0
17—Gu	F	9.8	3.74	26.2	—	—	22	4	42½	35	0	0
18—Si	F	10.2	3.49	29.2	15	—	11	3	35	35	0	0
19—Ka	F	10.2	3.98	25.6	—	—	26½	5	32½	65	0	0
20—Be	F	10.3	3.40	33.0	—	—	19½	4	42½	40	+	0
21—Es	M	10.7	4.00	27.3	5	—	23½	4	*	*	*	*
AVERAGE		8.3	3.51	23.9			25.0	4.52	42.5	51.8		
SAMPLE STANDARD DEVIATION		1.7	.53	4.9			9.8	1.7	8.7	10.3		
B. Treated Iron-Deficiency Anemia												
22—Cu	F	5.2	3.0	17.3	—	2,000 c.c. whole blood, 1 day	56½	4½	50	95	0	0

1—Ka	F	6.0	2.86	21.0	—	I.V., 6 days 0.6 Gm.	46½	8	77½	77½	+	+
2—Wa	F	6.7	2.90	23.1	—	I.V. 500 mg. 7 days	*	*	55	60	+	0
5—Li	M	8.5	—	—	—	Oral, 5 days	49½	5½	72½	65	+	0
23—Sc	M	10.0	3.98	25.1	—	2,000 c.c. whole blood, 5 days	21½	7	55	77½	+	+
19—Ka	F	11.5	4.28	27.0	—	Oral, 7 days	30½	2½	35	55½	*	*
17—Gu	F	11.5	3.97	29.0	—	Oral, 4 weeks	*	*	42½	32½	+	0
10—Vr	F	12.3	4.49	27.4	—	Oral, 4 weeks	29	5½	62½	60	+	0
20—Be	F	12.4	—	—	142	Regular oral, 1 year	24	6½	47½	52½	+	+
17—Gu	F	12.8	3.70	34.6	—	Regular oral, 4 months	12	3½	25	40	+	+
12—Ja	M	13.5	4.75	28.5	—	Intermittent oral, 8 months	27	2½	35	50	0	+
19—Ka	F	13.7	4.63	29.5	—	Intermittent oral, 1 year	20	6	35	75	0	0
23—Sc	M	14.8	4.87	30.4	—	2,000 c.c. whole blood and oral, 1 year	*	*	47½	45	*	*
C. Normal Controls												
Ro	M	14.5	4.98	29.2	—	—	16½	3	37½	37½	+	+
Go	M	14.4	4.43	32.5	—	—	18	7	42½	35	+	+
Ja	M	14.6	4.38	33.4	—	—	15	7	*	*	*	*
Ga	M	16.2	5.08	32.0	—	—	*	*	32½	47½	*	*
Se	M	14.5	4.40	33.0	—	—	16	5½	60	42½	+	+
AVERAGE		14.9	4.65									
SAMPLE		.4	.39									
STANDARD DEVIATION												
Hu	F	12.7	4.01	31.7	—	—	14	4	37½	32½	+	+
Ru	F	13.8	4.58	30.1	—	—	15	8	25	45	+	+
Ca	F	13.8	4.27	32.3	—	—	28	2½	50	30	+	+
Pi	F	13.4	4.43	30.3	—	—	13	4	25	42½	+	+
Ma	F	14.3	4.26	33.5	—	—	14	5	40	45	+	+
Ku	F	13.0	4.21	30.9	—	—	19½	6½	27½	45	+	+
Cr	F	12.1	3.99	30.4	—	—	17	6	25	52½	0	+
AVERAGE		13.3	4.25	31.6			16.9	5.32	36.6	41.36		
SAMPLE		1.1	.26	1.8			2.7	1.75	11.4	6.72		
STANDARD DEVIATION												

*= Specimen inadequate.

large variability in the appearance of the marrows of untreated patients is also illustrated in Figs. 4 and 5. There is no significant difference between the cellularity of the smears of anemic and control patients.

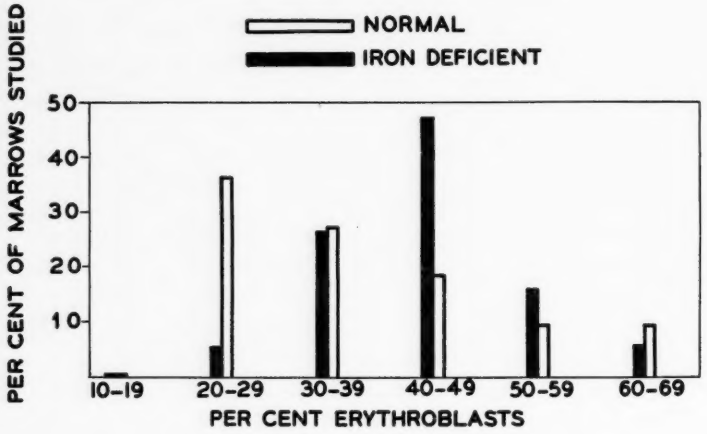

Fig. 2.—Histogram of the estimated percentage of erythroblasts in bone marrow sections from 11 normal controls and 19 patients with untreated iron-deficiency anemia.

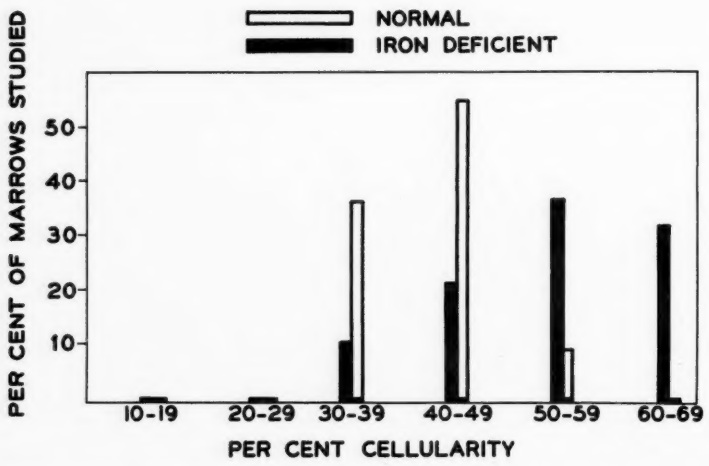

Fig. 3.—Histogram of the estimated cellularity of bone marrow sections from 11 normal controls and 19 patients with untreated iron-deficiency anemia.

The erythroblastic hyperplasia of the marrow of the anemic patients, when present, involves erythroblasts of all degrees of maturity. Occasionally the hyperplasia appears to be somewhat more marked in the more immature forms but this finding is by no means a constant one.

An attempt has been made in Fig. 6 to correlate the degree of anemia with the percentage of erythroblasts in smear or section or with the cellularity of the sections. It is evident that, in contrast to the findings of Scott,[1] no such correlation exists in our data. A similar lack of correlation is found to

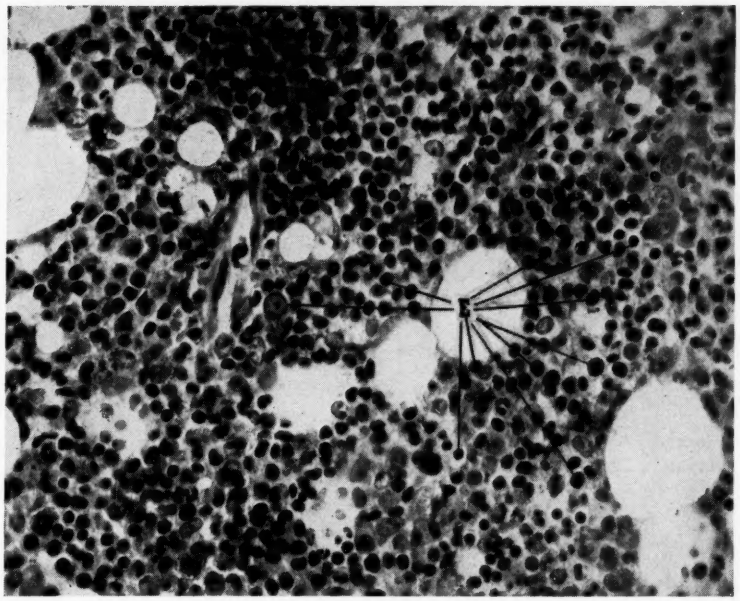

Fig. 4.—Section of the bone marrow of iron-deficient patient 10-Vr before treatment, showing that marked erythroblastic hyperplasia may be present. *E* indicates erythroblasts. (Hematoxylin-eosin-azure. ×500; reduced ¼.)

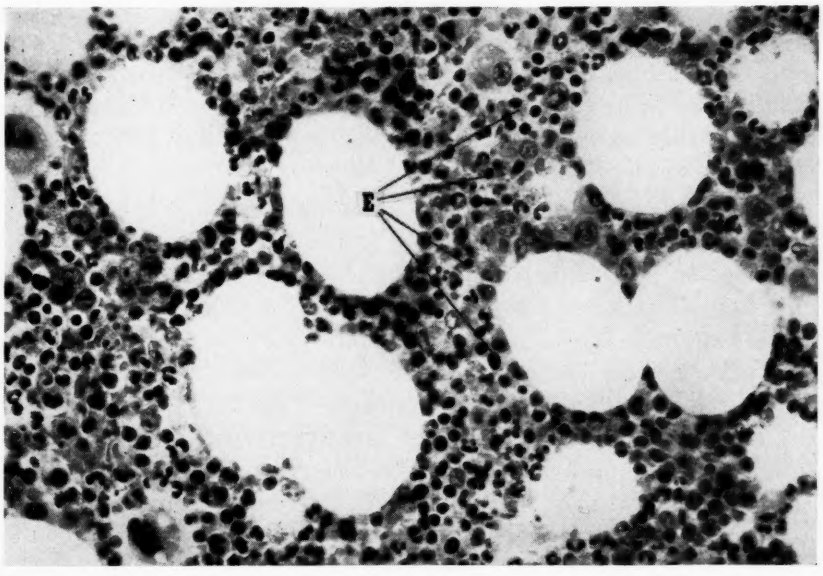

Fig. 5.—Section of the bone marrow of iron-deficient patient 11-Le before treatment, showing that the marrow may be entirely normal in appearance. *E* indicates erythroblasts. (Hematoxylin-eosin-azure. ×500; reduced ¼.)

exist between the degree of hypochromicity and erythroblastic activity of the marrow. We find no other differences between the histology of the marrows of normal and iron-deficient patients.

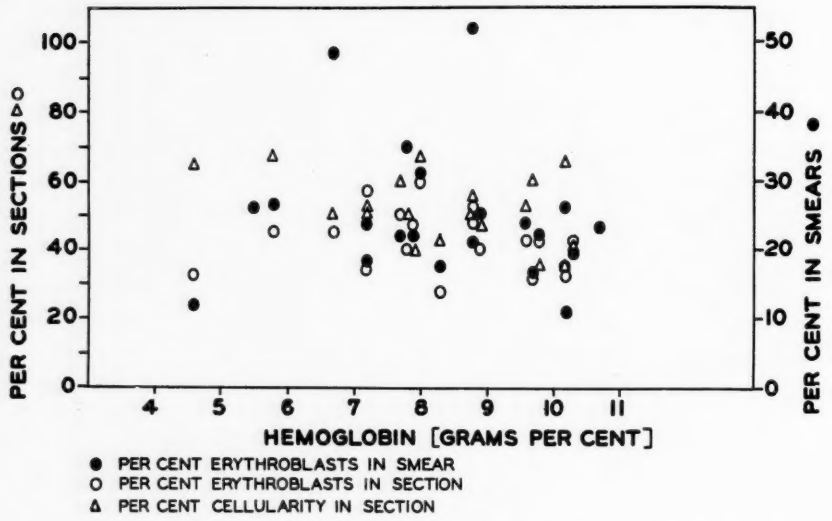
PER CENT IN SECTIONS
PER CENT IN SMEARS
HEMOGLOBIN [GRAMS PER CENT]
● PER CENT ERYTHROBLASTS IN SMEAR
○ PER CENT ERYTHROBLASTS IN SECTION
△ PER CENT CELLULARITY IN SECTION

Fig. 6.—Scattergram of the relationship of the histopathology of the marrow to the severity of untreated iron-deficiency anemia.

The patients from whom marrow specimens were obtained at various times after institution of therapy were too diverse to permit them to be considered as a group. The early response appears to be one of intensified erythroblastic hyperplasia. The magnitude of the response is roughly proportional to the severity of the anemia and diminishes as the anemia disappears, but even after many months of treatment some marrows are slightly more erythroblastic than those of the normal controls. It must be mentioned in this regard, however, that two of these patients (19-Ka, 20-Be) had continued to have excessively heavy menses.

It is evident from Table I that the interpretations of H.E.A. and iron-stained sections regarding the presence of stored iron are not always in agreement. Most often this discrepancy is due to the fact that greenish granules resembling hemosiderin are present in the H.E.A. sections but fail to give the Berlin blue reaction. For reasons given later we accept the iron stain as giving the more correct evaluation of the iron stores. According to this interpretation iron was found in all of the control marrows* but in none of the untreated iron-deficient patients. After treatment with intravenous iron or blood, stainable iron appeared in 3 of the 4 marrows examined but in only 3 of 6 receiving only oral iron. The only patient receiving intravenous therapy in whose marrow stainable iron was not detected had received 1,000 ml. of

*Through the courtesy of Captain R. J. Dern, Medical Corps, United States Army Reserve, we have had the opportunity of examining additional sternal marrow sections from 15 normal male volunteers from the Stateville Malaria Project. Stainable iron was absent in only 4 patients, each of whom had donated at least 4 pints of blood in the preceding year.

blood only one day prior to the biopsy in treatment of very severe anemia. The three patients who had received iron only by the oral route who had succeeded in storing stainable iron in the marrow had received treatment for four to twelve months. A fourth patient who had been on iron therapy for a similarly long period of time failed to store any stainable iron, as did the three cases who had received treatment for only five days to four weeks.

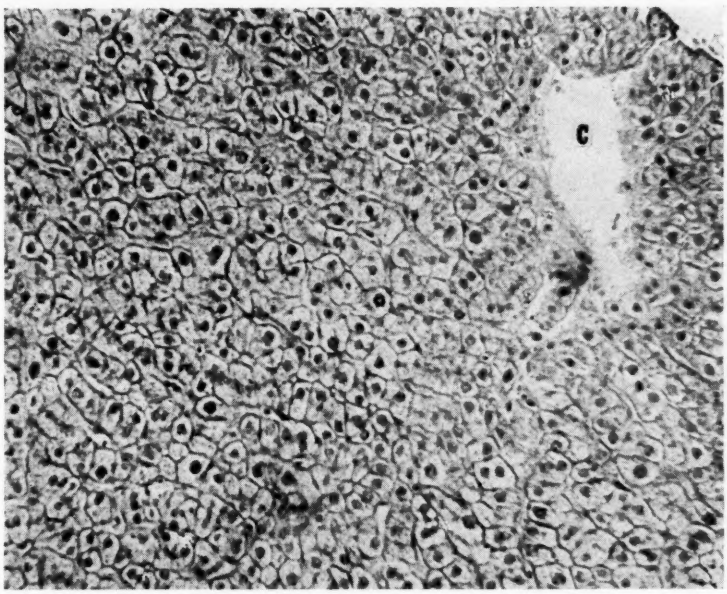

Fig. 7.—Liver biopsy of iron-deficient patient 1-Ka before treatment. No stainable iron is present. *C* indicates central vein. Stained for iron. (Hematoxylin and eosin counterstain ×250; reduced ¼.)

Livers.—Biopsies of the livers of two patients with untreated iron-deficiency anemia appeared normal histologically. No stainable iron stores could be demonstrated in either. From one of these patients 2 further biopsies were obtained, one after the administration of 500 mg. and one after administration of 2,300 mg. of iron intravenously. These biopsies demonstrate no change except for the deposition of iron exclusively in the littoral cells of the sinusoids, presumably in Kupffer cells (Figs. 7, 8, and 9). No extramendullary blood formation was observed. In a third diabetic iron-deficient patient, liver biopsies were obtained first after 2,200 ml. of blood and 30 mg. of iron had been given intravenously, and again after an additional 300 mg. of intravenous iron had been administered. The liver of this patient shows fatty change of the liver cells and chronic inflammation and fibrosis of the portal areas. In the first of the two biopsies, no iron could be demonstrated. The second biopsy, however, shows the characteristic distribution of iron in Kupffer cells. The fatty change and fibrosis did not change in the six-day interval between biopsies, and is believed to represent an independent pathologic process.

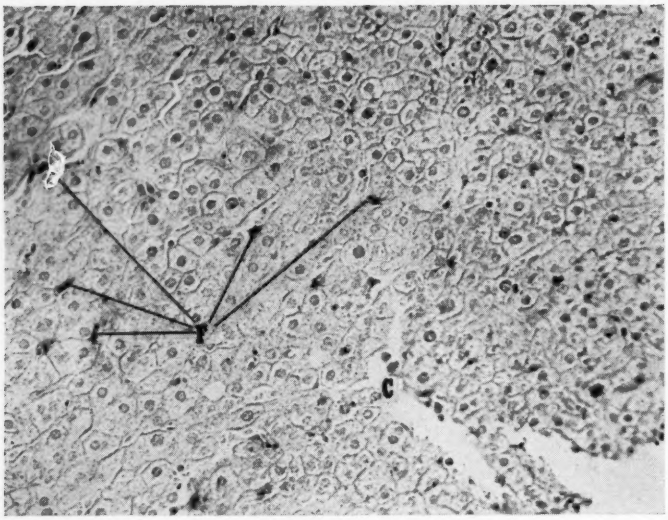

Fig. 8.—Liver biopsy of patient 1-Ka after intravenous administration of 500 mg. of iron. *F* indicates the blue-stained iron granules; *C*, the central vein. Stained for iron. (Hematoxylin and eosin counterstain. ×250; reduced ¼.)

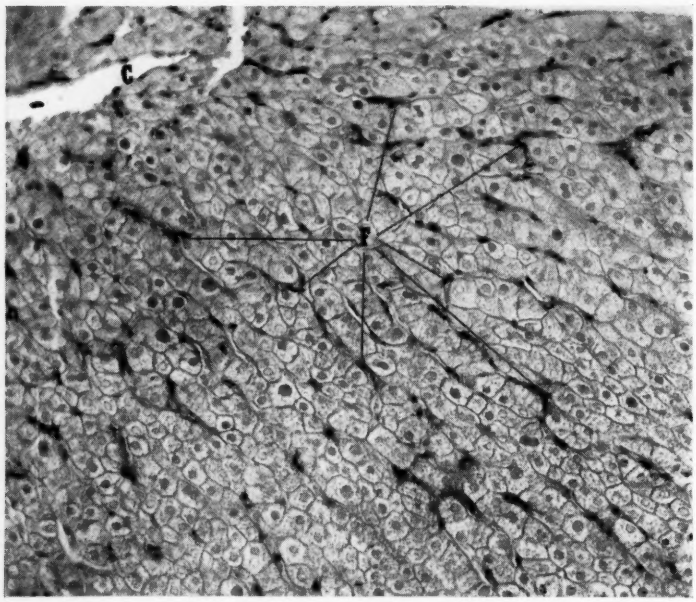

Fig. 9.—Liver biopsy of patient 1-Ka after intravenous administration of 2,300 mg. of iron. *F* indicates the blue-stained iron granules; *C*, the central vein. Stained for iron. (Hematoxylin and eosin counterstain. ×250; reduced ¼.)

DISCUSSION

The histopathology of bone marrow sections from a sizable series of iron-deficient patients has not been reported previously. The use of sections is believed to be superior to the use of smears because the original relationship between marrow elements is maintained and because preservation of the reticulum cells permits more reliable estimates to be made of the iron stores.[21, 22] By examination of sections as well as smears we are able to confirm the observation that the marrow of untreated iron-deficiency anemia is erythroblastic, but must agree with Stodtmeister that this tendency is not sufficiently great to be of any diagnostic value.

Study of the iron content of the marrow by histochemical means has proved highly rewarding. The marrow frequently contains granules which resemble hemosiderin but fail to give the highly specific Berlin blue reaction. Even after oxidation with 30 per cent hydrogen peroxide to "unmask" organically bound iron[29] no stainable iron could be demonstrated.[30] For this reason, and because such granules were occasionally found in marrows of severely and chronically iron-deficient patients (e.g., 7-Ga and 11-Le), we have concluded that they do not represent storage iron. The further finding, in agreement with that of Hutchison,[22] that we are sometimes able to stain nongranular iron stores, leads us to conclude that an iron stain should be employed if the iron stores are to be assessed in sections with a high degree of confidence. Stevens, Coleman, and Finch[19] state that they are able to detect hemosiderin with accuracy in unstained fresh marrow preparations. Unfortunately their technique does not permit permanent preservation of the material examined.

The iron content of the marrow, in contrast to its histological appearance, clearly distinguishes the iron-deficient from the normal patient. This observation is in agreement with those of Rath and Finch,[18] Stevens, Coleman, and Finch,[19] and Hutchison,[22] as opposed to the findings of Masshoff and Gruner.[20]

Finch and co-workers[32] have shown that stainable storage iron is available for hemoglobin synthesis. An important problem which has not received sufficient attention is whether this store may be repleted in iron-deficient patients by treatment with oral iron. Direct measurements in animals[31] and estimation by phlebotomy in man[32, 33] have indicated that such replacement of lost iron stores takes place very slowly or not at all. Histologic study of the marrows of orally treated iron-deficient patients has, however, only scant notice.[23] Although the number of biopsies we have been able to obtain on such patients has been limited, we have not been able to demonstrate the presence of iron in the marrows of any of 3 patients receiving oral therapy for four weeks or less, but it was demonstrable in three of four patients receiving oral iron for four to twelve months. In sharp contrast is the almost immediate, uniform appearance of stainable iron in the marrows and livers of patients receiving parental iron.

We believe that it is of considerable importance from a therapeutic point of view to realize that although slow, reaccumulation of storage iron may oc-

cur in the marrows of some iron-deficient patients treated by the oral route for prolonged periods of time. Because it is obviously highly desirable that such stores be repleted, oral therapy should never be discontinued as soon as the peripheral blood picture has returned to normal, but rather should be carried on for a period of many months in an attempt to replace the body's iron stores.

Adherence to this principle in the management of iron-deficient patients may help to reduce the high relapse rate of this disease.

SUMMARY AND CONCLUSIONS

Sections and smears of bone marrows from 23 iron-deficient patients have been studied both before and at various stages after initiation of treatment. Marrows from 12 normal patients served as controls.

The bone marrows of patients with untreated iron-deficiency anemia were, on the average, more erythroblastic and more cellular than the marrows of the normal controls, but variability in both groups was so great that the histopathology of the marrow cannot be considered diagnostic.

No appreciable correlation was found between the severity of the anemia and the degree of erythroblastic hyperplasia in the marrows of untreated patients.

The marrow responds to treatment by intensified hyperplasia, roughly proportional to the degree of anemia.

Stainable iron appeared in the marrow rapidly after treatment of iron-deficiency anemia with intravenous saccharated iron oxide, but more slowly or not at all after oral administration of iron.

Needle biopsies of the liver revealed no constant pathologic changes. After administration of intravenous saccharated iron oxide, storage appeared to take place exclusively in the Kupffer cells.

The importance of continuing oral iron therapy for a long time after the peripheral blood picture has returned to normal has been stressed.

We gratefully acknowledge the helpful advice of Dr. William F. Bethard and Dr. Leon O. Jacobson, and the technical assistance of Mrs. Melba Robson, Mrs. Dorothy Carter, and Miss Elizabeth Kelsey.

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THE VARIABILITY OF FECAL NITROGEN EXCRETION WITH LIQUID AND SOLID DIETS

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WITH THE TECHNICAL ASSISTANCE OF MARGARET BOLLIER, B.A., AND BETTY HACKELY, B.S.

INTRODUCTION

THE technique of nitrogen balance has been used with increasing frequency in the study of a variety of diseases. In such studies the analysis of fecal nitrogen has often been omitted because of lack of facilities for proper collection or analysis of feces, or because it was felt that sufficient accuracy was obtained by use of a standard value for fecal nitrogen. Thus, fecal nitrogen is assumed to be either ten per cent of the nitrogen intake or 1.3 Gm. per day, as proposed by Reifenstein, Albright, and Wells.[1] Each of these values has been criticized.[2, 3] The increasing use of liquid diets in nitrogen balance studies[4, 5] presents hazards resulting from lack of data on fecal nitrogen excretion with such diets.

To provide more information on fecal nitrogen excretion with liquid and solid diets data will be presented for sixty-one stool collection periods of 6.6 days average length during solid balance diets, and sixty-one stool collection periods of 7.4 days average length during liquid balance diets. The frequency of approximate prediction of fecal nitrogen excretion by various methods will be considered.

METHODS

In this report nitrogen balance data from the study of twenty-six patients will be considered. Twenty-five patients were men, one was a woman; ages ranged from 17 to 58 years. Of the twelve patients who received solid balance diets five were considered normal, three patients had chronic viral hepatitis, and four patients had Laennec's cirrhosis. Liquid diets were used in fourteen patients; five with acute hepatitis, one considered normal, and eight patients with moderate to severe malnutrition following trauma. No patient on either liquid or solid diet was demonstrated to have significant abnormality in gastrointestinal function.

Solid diets for thirty to sixty day studies were prepared and stored in a freezer until served to the patient. Four to six analyses for nitrogen with half of the day's diet as an aliquot were made during each study. The mean of these analyses is used as the value of the nitrogen intake. The standard deviation of the mean of the analyses was ± 1.95 per cent for the 24 solid diets used. Nitrogen content of solid diets calculated from standard food tables[6] averaged 8.7 per cent less than the analyzed nitrogen content.

Liquid diets were made daily and refrigerated until served. Four to six analyses for nitrogen were made during each two to four week study period. The mean analytical

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Received for publication, Oct. 22, 1953.

value was used as the figure for nitrogen intake. The standard deviation of the mean of the analyses was ± 2.20 per cent in thirty liquid diets used. Nitrogen content of liquid diets calculated from standard food tables[6] averaged 4.1 per cent more than the analyzed nitrogen content. After complete mixing, nitrogen of both solid and liquid diets was determined by the macro-Kjeldahl method.

Stool collections were begun the same day a balance study was started and made for arbitrary intervals. Stools were constantly refrigerated at 8° C. and aliquots taken after complete mixing for nitrogen determination by the macro-Kjeldahl method.

RESULTS

The number and duration of stool collections with liquid and solid diets are shown in Table I.

TABLE I. NUMBER AND DURATION OF STOOL COLLECTIONS ON SOLID AND LIQUID BALANCE DIETS

SOLID DIETS		LIQUID DIETS	
NUMBER	DURATION	NUMBER	DURATION
1	5 days	8	5 days
39	6 days	19	6 days
8	7 days	12	7 days
8	8 days	6	8 days
1	9 days	4	9 days
4	10 days	7	10 days
61	6.6 days average length	1	11 days
		4	12 days
		61	7.4 days average length

The relation of daily nitrogen intake to daily fecal nitrogen excretion with liquid and solid diets is shown in Fig. 1. In calculating the regression line depicted in Fig. 1 stool collection periods in which diarrhea was present are excluded, and all six collections from one patient with cirrhosis were excluded because of unusually high fecal nitrogen excretion. With these omissions there were 106 stool collection periods. The results give a correlation coefficient of 0.66, which is highly significant, $T = 12.0$. The formula for the regression line is $Y = 0.51 + 0.067X$, where Y is the daily fecal nitrogen in grams and X is the daily nitrogen intake in grams.

The mean daily fecal nitrogen was 1.49 Gm. for stool collections during solid diets and 1.69 Gm. during liquid diets.

DISCUSSION

In a nitrogen balance study one tries to select a value for fecal nitrogen which is most likely to be correct, realizing that in the absence of stool analysis some error may be introduced into the nitrogen balance calculation. A desirable range of error for any estimate would be variation of ±0.25 Gm. nitrogen from the arbitrary value chosen, and an acceptable variation from the estimate might be ±0.5 Gm. nitrogen. Data presented by Toscani and Whedon[3] suggest that there is relatively little variation in the fecal nitrogen when a constant nitrogen intake is maintained. If this were true, it might be possible in any long study to determine the fecal nitrogen for the first six or seven days of the study and use this value for the remainder of the balance program.

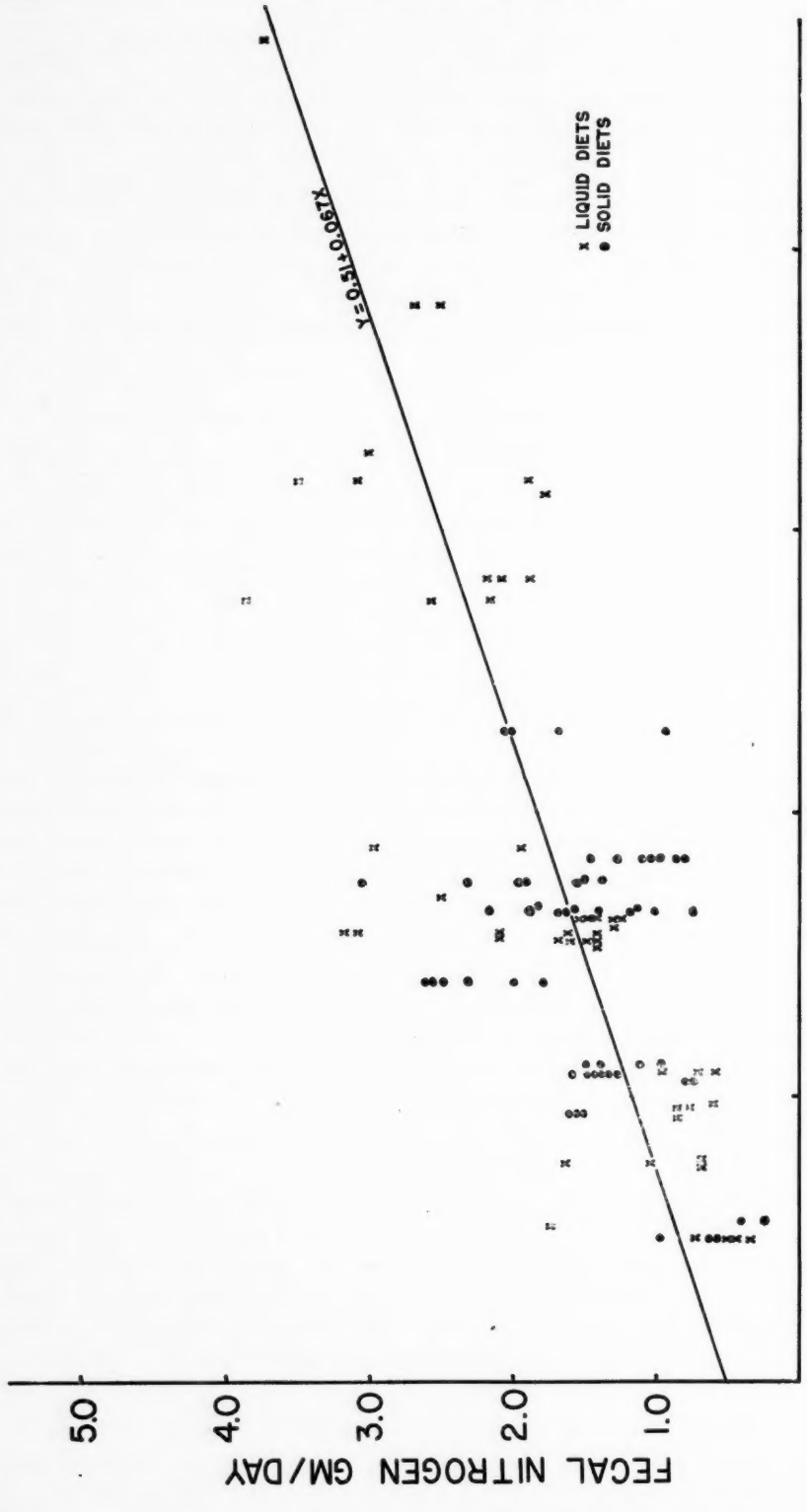

Fig. 1.—The relation of fecal nitrogen to nitrogen intake. The regression line calculated from the data is shown.

In Table II the use of an initial stool collection period to predict subsequent fecal nitrogen is considered. In addition, the standard methods of predicting fecal nitrogen and the regression value derived from the present study are also noted. The percentage of successful prediction of fecal nitrogen within the ranges specified is recorded for each method. Data from the present series and from a collected series[1, 3, 7, 8, 9] are analyzed.

It is apparent from Table II that estimation of fecal nitrogen by standard methods usually does not give accuracy of prediction within ±0.25 Gm. N, but does, in the majority of instances, give values within ±0.5 Gm. N of the analyzed fecal nitrogen. Use of the regression formula from the present series is not superior to standard methods in this regard. Utilization of an initial stool collection period for prediction of future fecal nitrogen values appears superior to other methods; however, this method is only applicable when nitrogen intake is fixed at a constant level.

TABLE II. PERCENTAGE OF SUCCESSFUL PREDICTION OF FECAL NITROGEN BY VARIOUS METHODS

	PRESENT SERIES	COLLECTED SERIES
Number of stool collection periods	106	229
Average length of stool collection periods	7.1 days	7.4 days
10% of intake ± 0.25 Gm. N	38%	38%
1.3 Gm. ± 0.25 mg. N	27%	41%
(0.51 + 0.067 × Intake) ± 0.25 Gm. N	37%	37%
First stool collection ± 0.25 Gm. N	43%	62%
10% of intake ± 0.5 Gm. N	59%	72%
1.3 Gm. ± 0.50 Gm. N	50%	76%
(0.51 + 0.067 × Intake) ± 0.5 Gm. N	68%	70%
First stool collection ± 0.5 Gm. N	73%	91%

For accurate nitrogen balance calculation stool analysis is essential. If stool collection or analysis is impossible the limitations of current methods of predicting fecal nitrogen should be appreciated. Conclusions drawn from minor changes in nitrogen balance are unwarranted unless all fecal nitrogen values are determined analytically.

CONCLUSIONS

Data are presented and a regression line derived for 106 stool collection periods during nitrogen balance studies at various levels of nitrogen intake. The likelihood of predicting fecal nitrogen excretion within defined limits by several methods is considered. On a fixed constant nitrogen intake the daily fecal nitrogen of an initial stool collection period serves best to predict subsequent fecal nitrogen excretion. Fecal nitrogen excretion is sufficiently variable to require analytic determination before minor changes in nitrogen balance can be interpreted.

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THE QUANTITATIVE MEASUREMENT OF HEPARIN-INDUCED LIPEMIA CLEARING ACTIVITY OF PLASMA

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WITH THE TECHNICAL ASSISTANCE OF PRIVATE FIRST CLASS DONALD V. BOWMAN

IN 1943 Hahn[1] discovered that injection of heparin into dogs displaying alimentary lipemia resulted in disappearance of the lipemic turbidity. Addition of heparin to lipemic plasma in vitro did not produce clearing. Anderson and Fawcett[2] reported that clearing ensued when plasma from a human subject who had received an injection of heparin was added to lipemic plasma in vitro.

In the present study it was found that synthetic fat emulsion could be substituted for lipemic plasma as a substrate for the action of the heparin-induced clearing factor. This serves as the basis for a quantitative method for the measurement of clearing activity.

METHODS

Blood samples were obtained from anesthetized rats by aortic puncture using a dry syringe. Blood samples were placed in an ice bath immediately after collection. Serum, or in the case of heparin-injected animals, plasma was separated by centrifugation in a refrigerated centrifuge. Serum or plasma samples were kept at 5° C. until they were used. In a few instances serum or plasma samples were stored in the frozen state for periods up to several days and then thawed at 5° C.

Heparin (Abbott, 10 mg./ml.) was injected intravenously. The fat emulsion used in this study contained 10 Gm. of sesame oil and 0.5 Gm. of Tween 60 per 100 ml. of 5 per cent dextrose. The emulsion* was prepared by high-pressure homogenization and had an average particle size of less than 1 micron. Immediately before use the emulsion was diluted to the appropriate concentration of fat (usually 1 per cent) with distilled water.

Turbidity of plasma-fat emulsion mixtures was measured in a Coleman, Jr. spectrophotometer at 700 mμ using micro-cuvettes.

RESULTS

Fig. 1 gives the results of an experiment in which 0.1 ml. of 1 per cent fat emulsion was added respectively to 1 ml. of serum from an untreated rat and 1 ml. of plasma from a rat which had received 0.5 mg./kg. of heparin intravenously fifteen minutes previously. This demonstrates that plasma from the heparin-injected animal causes clearing whereas that from the untreated animal does not. Most of the clearing occurs during the first twenty minutes after addition of the fat emulsion.

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The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

Received for publication, Oct. 22, 1953.

*Dr. Douglas V. Frost of Abbott Laboratories, North Chicago, Ill., kindly supplied this emulsion.

The effect of temperature on the stability and activity of the clearing factor was studied in the following way. Samples of plasma from rats which had received 10 mg./kg. of heparin intravenously ten minutes before the blood was drawn were pooled. One milliliter samples of this pooled plasma were placed in cuvettes, and these were placed in water baths maintained at various temperatures. At intervals samples were removed from the various baths, brought to 25° C., and 0.1 ml. of 1 per cent fat emulsion was added to each

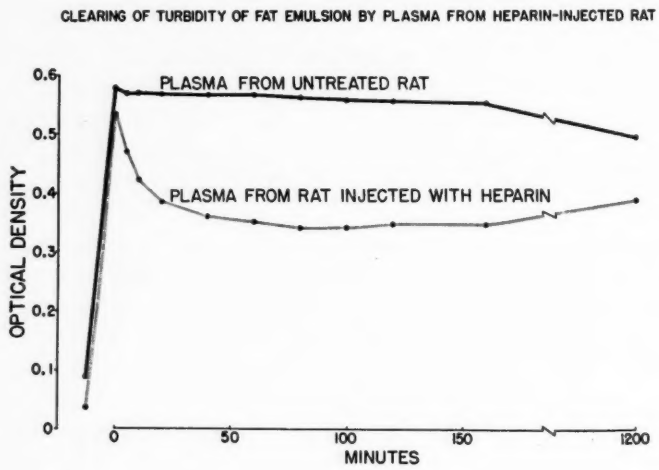

Fig. 1.

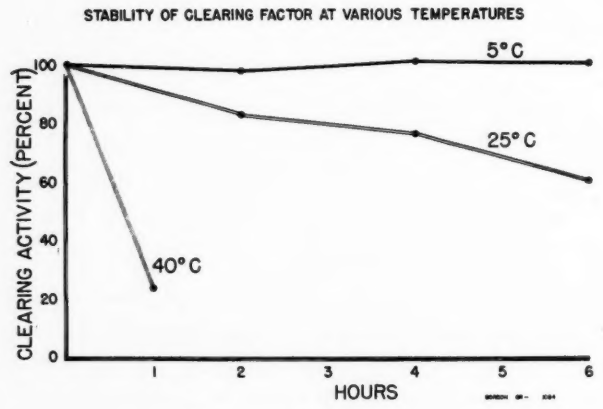

Fig. 2.

cuvette. Optical density was determined immediately after addition of the fat emulsion and after sixty minutes of incubation at 25° C. The results are presented in Fig. 2. Clearing activity, measured as fall in optical density during sixty minutes, is expressed as per cent of the original activity of the pooled sample. Each point on the graph represents the mean of duplicate determinations. At 5° C. there was no loss in activity in six hours. At 25° C.

there was approximately 30 per cent loss of activity in six hours and at 40° C., 80 per cent loss in one hour. This experiment demonstrates that the clearing activity of rat plasma is thermolabile but is stable for at least six hours at 5° C.

The same batch of pooled plasma was tested for clearing activity using various temperatures of incubation. Clearing activity was measured as the reduction in optical density during sixty minutes of a mixture of 1 ml. of plasma and 0.1 ml. of 1 per cent fat emulsion. The results are presented in Fig. 3. Maximal activity occurred at 20° to 25° C., falling off sharply above or below this temperature. All subsequent tests were performed at 25° C.

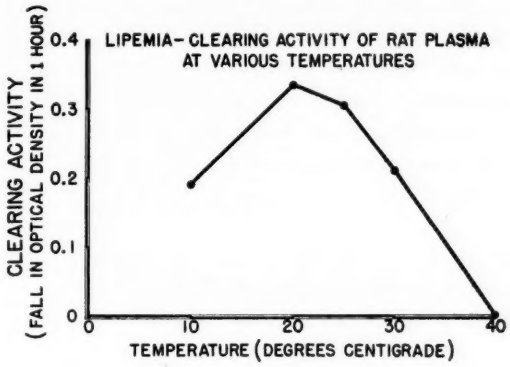

Fig. 3.

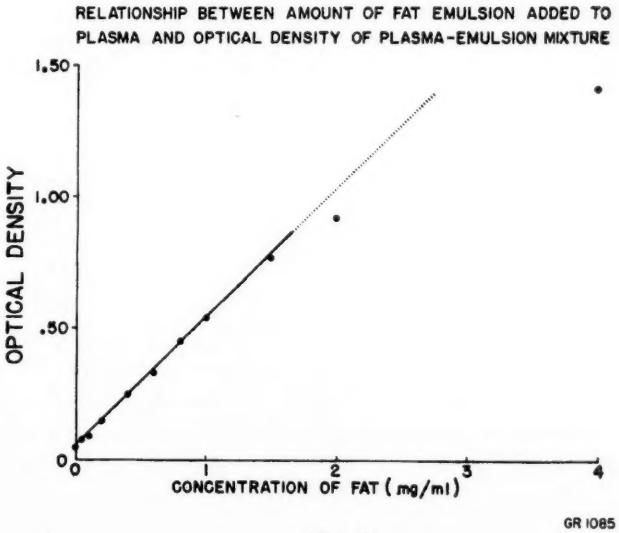

Fig. 4.

The relationship between concentration of fat emulsion and optical density of plasma-fat emulsion mixtures was determined by adding 0.1 ml. of fat of various concentrations to 1 ml. samples of plasma from an untreated animal (no clearing activity). The results appear in Fig. 4. Concentration of fat is expressed as mg./ml. of the plasma-fat emulsion mixture. Up to an optical

density of about 0.75, corresponding to a fat concentration of 1.5 mg./ml., the fat concentration and optical density are rectilinearly related. Above this concentration, increase in fat concentration produces decreasing increments of optical density. In the standard clearing test, as described below, 0.1 ml. of 1 per cent fat emulsion is added to 1 ml. of plasma giving a fat concentration of 1 mg./ml. of plasma-fat emulsion mixture.

To determine the effect of concentration of fat in the incubation mixture on clearing activity an experiment was conducted using various dilutions of the stock 10 per cent fat emulsion. To 1 ml. of plasma 0.1 ml. of 1, 2, or 4 per cent fat emulsion was added and the optical density was measured immediately and after sixty minutes of incubation at 25° C. Two plasma samples were used, from rats which had received 10 mg./kg. or 1 mg./kg. of heparin intravenously ten minutes before taking the blood samples. The results appear in Fig. 5. The amount of clearing, as measured by the fall in optical density in sixty minutes, was the same with 1 mg./kg. of heparin as with 10 mg./kg. of heparin, and was the same with 2 per cent as with 1 per cent fat emulsion,

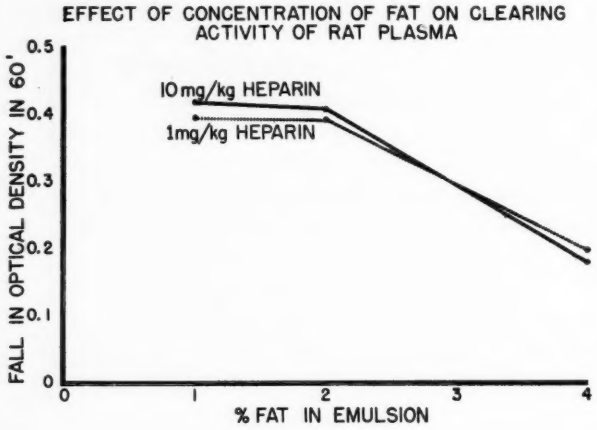

Fig. 5.

but was apparently less with 4 per cent emulsion. This apparent falling off of activity with the higher fat concentration can be attributed, at least in part, to the fact that at this concentration of fat the optical density is no longer a linear function of fat concentration, as pointed out above. This experiment indicates that the failure to obtain higher activity with the higher dose of heparin is not due to inadequate substrate acting as a limiting factor.

To determine the relation between concentration of clearing factor and amount of clearing the following experiment was carried out. Plasma from heparin-injected rats was mixed in varying proportions with plasma from untreated rats (no clearing activity) and the optical density of these mixtures was measured immediately and at 5-minute intervals for 1 hour after addition of 0.1 ml. of 1 per cent fat emulsion. The results are presented in Fig. 6. When clearing activity is expressed as the decrease in optical density during

sixty minutes, the relationship between amount of clearing factor and activity is hyperbolic with a steep rise at the low concentrations and a long plateau at higher concentrations. If, however, the values from the same determinations are plotted as concentration of clearing factor against activity expressed as the reciprocal of the length of time required for a fixed fall in optical density (0.15 optical density units*) the relationship is essentially linear over the entire concentration range. It is obvious that the use of the first method of expressing activity may lead to errors.

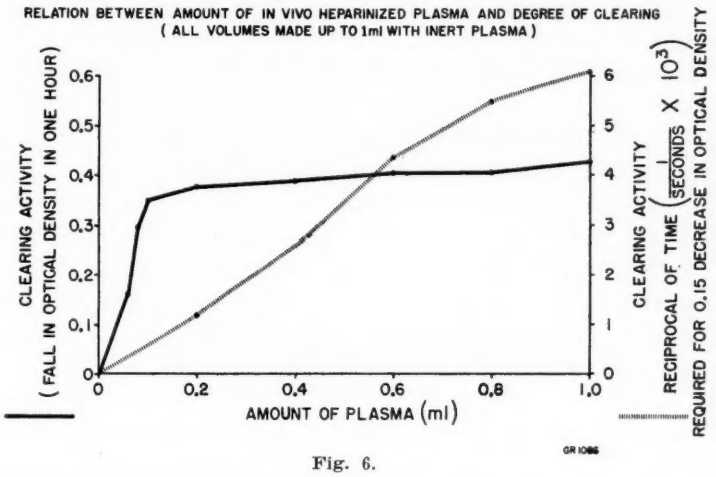

Fig. 6.

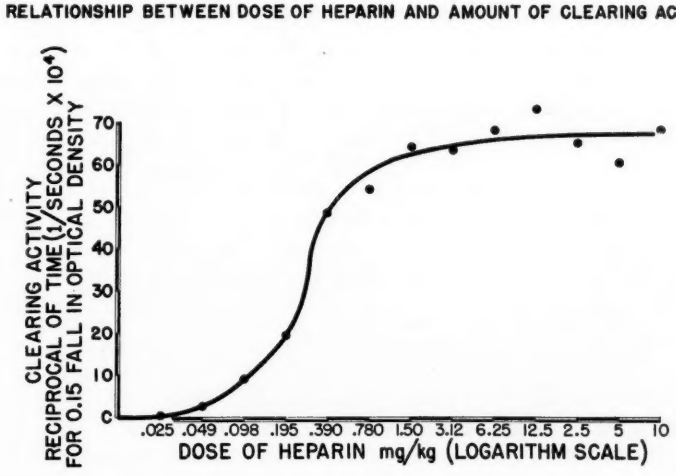

Fig. 7.

The relationship between the dose of heparin injected and the amount of clearing activity was studied by injecting various doses of heparin intravenously in rats and taking blood samples from them ten minutes after in-

*This value (0.15 optical density units) was selected because it represents approximately 50 per cent of maximal clearing.

jection. Three rats were used at each dose level. The mean values are presented in Fig. 7. The dose of heparin is plotted on a logarithmic scale and clearing activity is expressed as the reciprocal of the time required for a fall in optical density of 0.15. Between 0.05 and 1.0 mg./kg., activity increased with increasing dose. Above this dosage level, no further increase in clearing activity was produced.

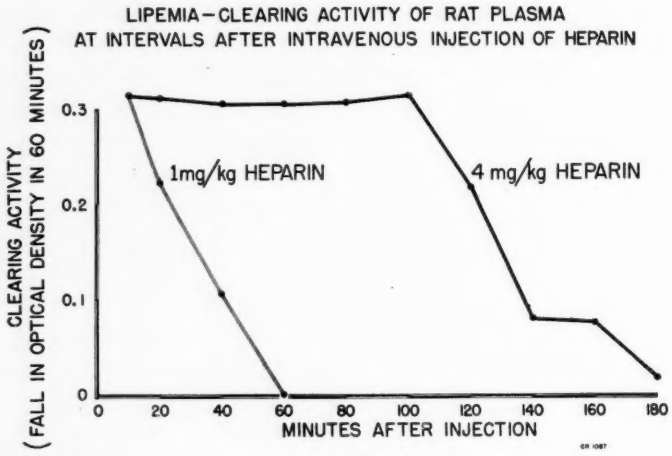

Fig. 8.

In Fig. 8 are presented the results of an experiment to show the length of time that clearing factor remains in the plasma after an injection of heparin. Ten minutes after injection the amount of clearing factor in the plasma is the same with a 1 mg./kg. dose of heparin as with a 4 mg./kg. dose, confirming the results presented in Fig. 5. With 1 mg./kg., however, the clearing activity rapidly falls off and has returned to zero at the end of one hour after injection, whereas with 4 mg./kg. the clearing activity remains high for two hours and does not return to zero until after three hours.

STANDARD PROCEDURE FOR DETERMINATIONS OF CLEARING ACTIVITY

On the basis of the experiments presented previously, the following standard procedure for determination of the clearing activity of plasma has been adopted:

Draw blood in a dry syringe. Place blood sample in ice bath immediately. Separate plasma or serum by centrifugation in a refrigerated centrifuge. Pipette off plasma or serum and store it at 5° C. until determination is carried out; or store in frozen state and thaw at 5° C. Transfer 1 ml. of plasma or serum to micro-cuvette. Place cuvette in 25° C. water bath for three minutes. Take control optical density reading (700 mμ, Coleman, Jr. spectrophotometer), using water as a blank. Add 0.1 ml. of 1 per cent fat emulsion, mix rapidly by inversion and take initial reading immediately. Since clearing begins as soon as fat emulsion is added, it is important that the first reading be taken as soon after adding the fat as possible. Replace cuvette in 25° C. bath. Take optical density readings at 5, 10, 20, 30, 40, 50, and 60 minutes. Continue readings until the optical density has fallen 0.15 or more units below that of the initial reading. If the optical density has fallen more than 0.15 in five minutes, repeat the test leaving the cuvette in the spectrophotometer

and, using a stop watch, record time in seconds when optical density has fallen 0.15. If optical density falls less than 0.15 in one hour, end the test at this time, recording the fall in optical density that has occurred.

Calculation:

$$\text{Clearing activity (units/ml.)} = \frac{1{,}000}{\text{time (seconds) for 0.15 fall in O.D.}}$$

The factor of 1,000 is arbitrarily used so that units will be whole numbers. Time required for 0.15 fall in optical density is obtained by interpolation of the observed values, or, in case the fall is less than 0.15 in one hour, by extrapolation.

DISCUSSION

The method described above has been used to determine the clearing activity of several hundred plasma samples from rats, rabbits, and human subjects. The agreement between duplicate determinations is good, usually showing less than 5 per cent variation.

The thermolability of clearing factor in rat plasma makes it necessary to keep samples at a low temperature before they are tested. The clearing factor of human postheparin plasma is somewhat more thermostable, showing about 40 per cent loss of activity in six hours at 40° C.

Because of the wide current interest in the subject of the effect of heparin on lipid and lipoprotein metabolism,[3, 4] a quantitative method for determination of heparin-induced clearing activity should prove valuable. The method is now in use in this laboratory in studies on the effect of heparin on the metabolic fate of intravenously administered synthetic fat emulsions.

The fact that neutral fat can serve as a substrate for the clearing factor indicates that the conversion of high S_f to low S_f lipoproteins as reported by Boyle and co-workers[5] is not the sole mechanism of action of the clearing factor.

SUMMARY AND CONCLUSIONS

A method is described for quantitative measurement of clearing activity based upon the reduction in turbidity (measured as a fall in optical density) of a mixture of plasma and synthetic fat emulsion.

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BREAK-THROUGH OF INTESTINAL ACTIVITY FROM INHIBITION BY METHANTHELINE BROMIDE

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ALTHOUGH Youmans has described the "break-through" of intestinal activity from the inhibition of continuously administered epinephrine,[1, 2] this phenomenon has not been reported previously in connection with methantheline bromide (Banthine). The observation that doses of Banthine repeated over a short period of time were progressively less effective in inhibiting canine intestinal motility led to an investigation of this phenomenon.

METHODS

Four dogs with Thiry-Vella fistulas of the jejunum and 2 with Thomas cannulas in the duodenum were used. Records of intestinal activity were made from thin rubber balloons measuring approximately 2 by 4 cm., introduced into the intestinal lumen and partially filled with 2 to 3 ml. of air. Kymographic records were made by using rubber-covered tambours to activate writing points. The animals were fed 500 Gm. of raw horse meat at the beginning of each test.

Banthine† dissolved in 0.9 per cent solution of sodium chloride was injected intravenously. In some tests single intravenous injections of the solution were given, usually in a dose of 0.5 mg. per kilogram of body weight, while in others the Banthine was administered continuously by an electrically driven syringe, which delivered 0.67 ml. of solution per minute. The dose of Banthine in the latter tests varied from 0.1 to 0.3 mg. per kilogram per minute. Control observations were made on the mean amplitude of intestinal contractions after injection of 0.9 per cent solution of sodium chloride. Similar measurements were made after the Banthine was injected and records were made of the time required for the contractions to return to 50 per cent of the amplitude seen during the control period.

RESULTS

After an initial single intravenous injection of Banthine, rapid and complete inhibition of rhythmic contractions of the intestine occurred. This effect persisted for approximately twenty to twenty-five minutes and was followed by a gradual return of contractions equal to those seen during the control period. Subsequent injections of the same or larger doses were followed by periods of inhibition which were of much shorter duration (Table I).

Two or three minutes after the continuous intravenous injection of Banthine at the rate of 0.1 mg. per kilogram per minute was begun, a similar cessation of intestinal activity took place. This usually lasted about fifteen to thirty minutes and was followed by a gradual return of rhythmic contractions

Received for publication, Oct. 23, 1953.

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†Kindly supplied by Dr. I. C. Winter of G. D. Searle & Co., Skokie, Ill.

of the intestine despite the fact that the infusion of Banthine was continued. Once the intestinal activity had reappeared, doubling or trebling the rate of injection of Banthine produced little further change (Fig. 1). This series of events was observed in 3 animals receiving, respectively, 131, 145, and 146 mg. of Banthine per test.

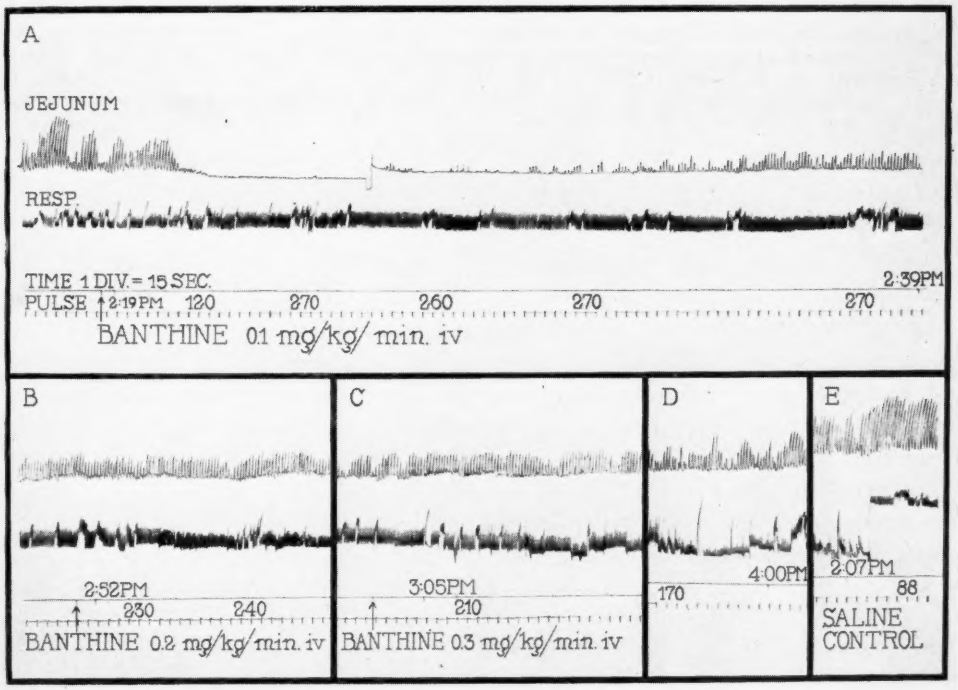

Fig. 1.—Kymographic tracing reversed from smoked-drum record, retouched and edited, from a 12.4 kilogram dog with a balloon in a Thiry-Vella fistula, recording jejunal activity. In *A*, continuous intravenous injection of Banthine was begun. In *B* and *C*, the rate of continuous injection was doubled and trebled, respectively. *D* indicates the activity thirty-six minutes after cessation of Banthine administration, total dose being 145 mg. *E* indicates the preliminary control period with continuous intravenous injection of 0.9 per cent solution of sodium chloride at the same rate as Banthine. Similar tracings were obtained from 2 other dogs under similar conditions, each with a Thomas cannula in the jejunum.

TABLE I. EFFECT OF REPEATED SINGLE INJECTIONS OF BANTHINE ON MOTOR ACTIVITY OF THIRY-VELLA FISTULAS IN DOGS

	MEAN TIME (MINUTES) AFTER EACH INJECTION FOR INTESTINAL CONTRACTIONS TO RETURN TO 50 PER CENT OF CONTROL AMPLITUDE			
DOG	1ST	2ND	3RD	4TH
1	25.3	18.0	1.0	1.0
2	20.5	4.5*	1.0*	
3	24.5	6.5	1.0	

*All injections consisted of Banthine given intravenously in doses of 0.5 mg. per kilogram of body weight except in those instances indicated with an asterisk (*) which included doses of 1.0 and 5.0 mg. per kilogram in some experiments.

CONCLUSIONS

Canine intestinal motility, measured in Thiry-Vella fistulas or in lateral fistulas of the small intestine, is promptly and temporarily suppressed when

appropriate doses of Banthine are given intravenously. If doses are repeated, or if the drug is given continuously, the intestinal musculature becomes refractory to the inhibiting effect of the drug. This sequence of events is similar to that which occurs after similar administration of epinephrine. These data provide no information on the mechanism involved in this phenomenon.

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THE EFFECT OF CIGARETTE SMOKING ON VENTILATORY FUNCTION IN PATIENTS WITH BRONCHIAL ASTHMA AND OBSTRUCTIVE PULMONARY EMPHYSEMA

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SYLVIA ITKIN AND FRED DRIMMER

THE purpose of this study was to investigate the acute effects of tobacco smoking on ventilatory function in patients with bronchial asthma and the bronchospastic types of pulmonary emphysema, chronic bronchitis, and bronchiectasis, as well as a group of normal subjects. The immediate effect of smoking two cigarettes was studied by Juurup and Muido[1] who found no change in oxygen consumption, ventilation, or respiratory rate. The long-term results of smoking on respiratory function of normal individuals indicated either no effect[2] or a decrease in vital capacity.[3, 4, 5] In this investigation, habitual smokers were employed to determine whether the impairment of respiratory function was produced by the smoking of three cigarettes, especially in respect to the production of bronchospasm.

METHODS

A total of 91 patients with well-documented chronic nontuberculous pulmonary disease was selected from office and clinic practice as subjects for this study. There were 25 women ranging in age from 16 to 67 years, with a mean of 47.0 years, and 66 men ranging from 29 to 77 years of age with a mean of 58.0 years. The disease entities for which they were being treated included intractable bronchial asthma and hypertrophic pulmonary emphysema of the bronchospastic type. Most of these subjects exhibited varying degrees of pulmonary emphysema associated with clinically significant bronchospasm at some time in the course of their disease.

A second group of 27 subjects consisting of medical students and technicians with no evidence of pulmonary disease were additionally studied. There were 20 men ranging in age from 23 to 52 years with a mean of 31.0 years, and 7 women ranging from 25 to 45 years of age with a mean of 30.6 years. Because of the disparity in clinical symptomatology and age which existed between the two groups, no direct comparison was attempted. Nevertheless, it was of interest to observe the effect of tobacco smoking in a group of individuals with no evidence of pulmonary emphysema or bronchospasm. Since it was not the intention of this investigation to test the side effects resulting from the absorption of nicotine, all the subjects chosen for this experiment were mild to moderate smokers, habitually smoking between 5 and 20 cigarettes per day.

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Received for publication, Oct. 27, 1953.

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Ventilatory function studies were performed on a closed-circuit Benedict-Roth spirometer. A motor blower on the inspiratory side served to overcome the slight resistance of one-inch tubing and soda lime. Straight-line graphs of pulmonary ventilation were obtained by continuous replacement of the oxygen consumed by the subject and removed from the bell, by the method described by Eckman and Barach[6] in which the oxygen content of the bell remains relatively constant (20.8 per cent ± 0.5) without artificial disturbance of the patient's pulmonary ventilation. Simultaneous tracings by a ratchet-counter attachment recorded the minute-ventilation and maximum breathing capacity. The open-circuit method described by Darling[7] was used in determining the functional residual volume, employing the Scholander[8] apparatus for the analyses of gas samples. These studies were performed in duplicate on each subject.

Subjects and patients were requested not to smoke or take any medication on the day of the test. The determinations of lung volumes, including vital capacity, expiratory reserve and residual volume were obtained while comfortably seated in an armchair two to three hours postprandially, and after being rested in the laboratory for thirty minutes prior to the control study. In addition, the minute pulmonary ventilation breathing air and 100 per cent oxygen over a seven-minute period, and the voluntary maximum breathing capacity were recorded, and all values were expressed as BTPS. These control studies were followed by the smoking of 3 cigarettes at a leisurely pace, approximately 30 minutes; and after a ten-minute rest period, the studies were repeated. Sixty-five of the 91 patients were retested following the inhalation of 0.3 ml. of 2.25 per cent racemic epinephrine.* A standard rest period of ten minutes was allowed between tests to avoid effects resulting from fatigue.

RESULTS

Patient Series.—Statistical analyses of the total lung volumes and their subdivisions in 91 patients before and after smoking three cigarettes is presented in Table I. A slight increase was found in the mean vital capacity, expiratory reserve air, and maximum breathing capacity after smoking. The mean resting pulmonary ventilations during inhalation of both air and oxygen fell slightly in 35 patients tested. The residual volume and the ratio of residual volume to total lung volume also showed a slight decrease. The percentage of nitrogen present in the alveolar gas samples after seven minutes of breathing 100 per cent oxygen, used as a measure of intrapulmonary gas mixing, showed a mean decrease of less than 0.5 per cent occurred after smoking. In appraising the significance of these small changes from the control level after smoking, t values were calculated for each ventilatory test. In order that the probability of a chance occurrence be less than 0.02, only values of t exceeding 2.5 were considered as showing a significant change. The application of these criteria to the various tests performed on the patient group indicated that the changes in mean values occurring after smoking could not be considered significant.

Of the 91 patients studied, increased coughing accompanied the smoking of 3 cigarettes in 38 subjects. When the incidence of coughing provoked by smoking was correlated with ventilatory studies, the results indicated a favorable effect in some of the cases in which elimination of secretions took place. Thus, a significant increase in the vital capacity and maximum breathing capacity was recorded in 9 patients, and in 12 a significant reduction in pulmonary ventilation took place. Only 5 patients showed a decrease in vital

*Supplied under the trade name Vaponefrin.

TABLE I. EFFECT OF CIGARETTE SMOKING ON VENTILATORY FUNCTION

TEST	IN 91 PATIENTS WITH NONTUBERCULOUS PULMONARY DISEASE			IN 27 NORMAL SUBJECTS		
	BEFORE	AFTER	PER CENT CHANGE	BEFORE	AFTER	PER CENT CHANGE
Vital capacity (L.)						
1. Mean	2.56	2.61	+2.9	4.45	4.47	0
2. Range	0.89 - 4.95	1.03 - 4.83	-26.0 : +61.0	2.20 - 5.76	2.36 - 6.20	-13.5 : +9.5
3. S. D.	0.76	0.77	12.65	0.96	0.98	4.67
Expiratory Reserve Air (L.)						
1. Mean	0.79	0.80	+5.2	1.8	1.7	-0.02
2. Range	0.13 - 1.66	0.07 - 1.8	-63.6 : +151.7	0.87 - 3.13	0.86 - 3.17	-58.0 : +52.8
3. S. D.	0.39	0.37	31.6	0.53	0.52	28.0
Maximum Breathing Capacity (L. per minute)						
1. Mean	35.4	36.4	+5.2	91.2	96.5	+8.2
2. Range	13.1 - 72.6	12.1 - 91.1	-36.6 : +146.7	55.3 - 135.5	59.8 - 140.0	-19.3 : +35.9
3. S. D.	13.3	14.6	26.5	25.3	25.1	16.3
Pulmonary Ventilation-Air* (L. per minute)						
1. Mean	10.7	10.2	-6.1	7.67	7.71	+1.2
2. Range	5.8 - 17.1	6.1 - 19.8	-26.7 : +31.2	4.3 - 10.8	4.4 - 11.3	-3.5 : +4.6
3. S. D.	2.34	2.26	12.5	2.89	3.02	3.05
Pulmonary Ventilation-Oxygen* (L. per minute)						
1. Mean	8.9	8.4	-6.9	9.1	8.8	-2.8
2. Range	5.7 - 13.5	5.4 - 13.7	-34.0 : +12.9	8.7 - 9.5	7.5 - 9.7	-19.4 : +11.3
3. S. D.	1.56	1.68	12.2	0.36	0.99	12.7
Residual Volume* (L.)						
1. Mean	3.6	3.5	-3.2	1.7	1.8	+8.3
2. Range	1.7 - 5.9	1.8 - 5.4	-33.0 : +31.0	1.5 - 1.9	1.7 - 2.1	-8.1 : +18.8
3. S. D.	1.11	1.17	13.9	0.21	0.20	14.4
Residual Volume / Total Capacity (%)						
1. Mean	58.5	56.3	-2.2	23.2	21.25	-1.5
2. Range	40.1 - 75.1	38.3 - 75.5	-10.9 : +6.0	21.7 - 24.7	17.2 - 25.3	-7.5 : +3.6
3. S. D.	10.16	11.46	4.1	2.14	5.7	5.3
Index of Intra-Pulmonary Mixing* (Alveolar Nitrogen %)						
1. Mean	10.9	10.5	-0.5	1.9	2.2	+0.4
2. Range	1.5 - 34.6	1.47 - 36.3	-15.7 : +13.7	1.3 - 2.5	1.3 - 3.2	-0.4 : +1.8
3. S. D.	9.1	8.6	5.6	0.59	0.91	1.29

*Performed on 35 patients and 4 normal subjects.

TABLE II. THE COUGH RESPONSE TO SMOKING AND ITS CORRELATION WITH VENTILATORY FUNCTION

NO. OF CASES	SIGNIFICANT INCREASE IN COUGH	VITAL CAPACITY			MAXIMUM BREATHING CAPACITY		
		INCREASE	NO CHANGE	DECREASE	INCREASE	NO CHANGE	DECREASE
91	38	9	24	5	9	21	8

capacity and 8 showed a fall in maximum breathing capacity. As shown in Table II, the majority of the patients who had an increase in coughing as a result of tobacco manifested no change in vital capacity or maximum breathing capacity.

Sixty-five of the 91 patients were retested after the inhalation of a bronchodilator aerosol, i.e., 0.3 ml. of Vaponefrin. The mean per cent change in vital capacity, which rose 2.88 per cent after smoking, increased an additional 10 per cent after Vaponefrin. Similar increases in the expiratory reserve volume and the maximum breathing capacity followed the inhalation of a bronchodilator aerosol. The data presented in Table III indicate a high degree of significance with all the t values exceeding 2.5. The probability

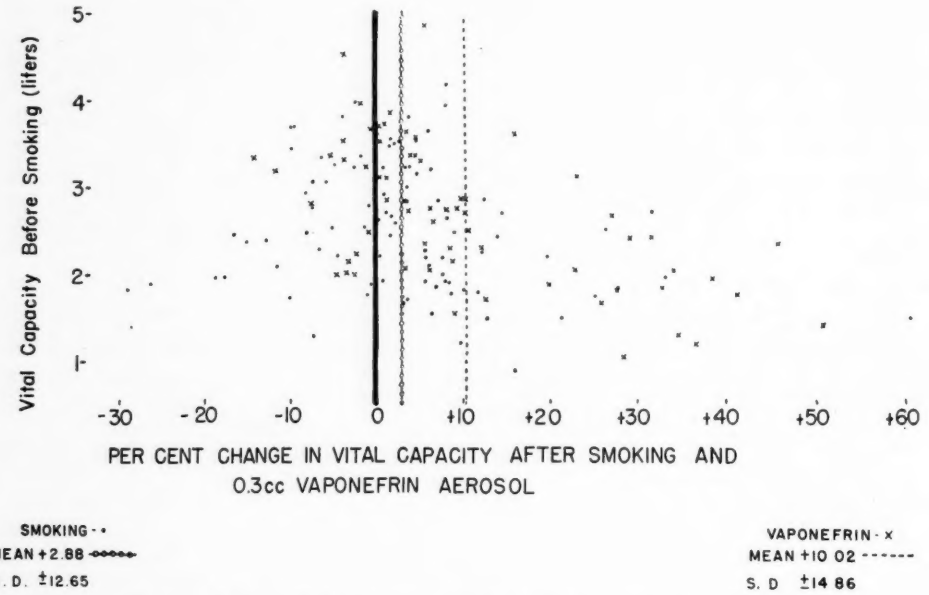

Fig. 1.—Change in the vital capacities of 91 patients with chronic bronchopulmonary disease after smoking 3 cigarettes and after 0.3 ml. of Vaponefrin aerosol expressed as per cent change from the control level.

TABLE III. EFFECT OF VAPONEFRIN (0.3 ML.) ON THE VENTILATORY FUNCTION OF PATIENTS WITH BRONCHOPULMONARY DISEASE AFTER SMOKING

TEST	NO. OF CASES	AFTER SMOKING (PER CENT CHANGE)	T VALUES	AFTER VAPONEFRIN (PER CENT CHANGE)	T VALUES
Vital Capacity (L.)					
Mean	65	+2.9	1.9	+10.0	5.4
S. D.		12.7		14.9	
Expiratory Reserve Air (L.)					
Mean	65	+5.2	0.1	+15.5	3.6
S. D.		31.6		34.3	
Maximum Breathing Capacity (L. per minute)					
Mean	65	+5.2	1.2	+18.5	7.6
S. D.		26.5		19.6	

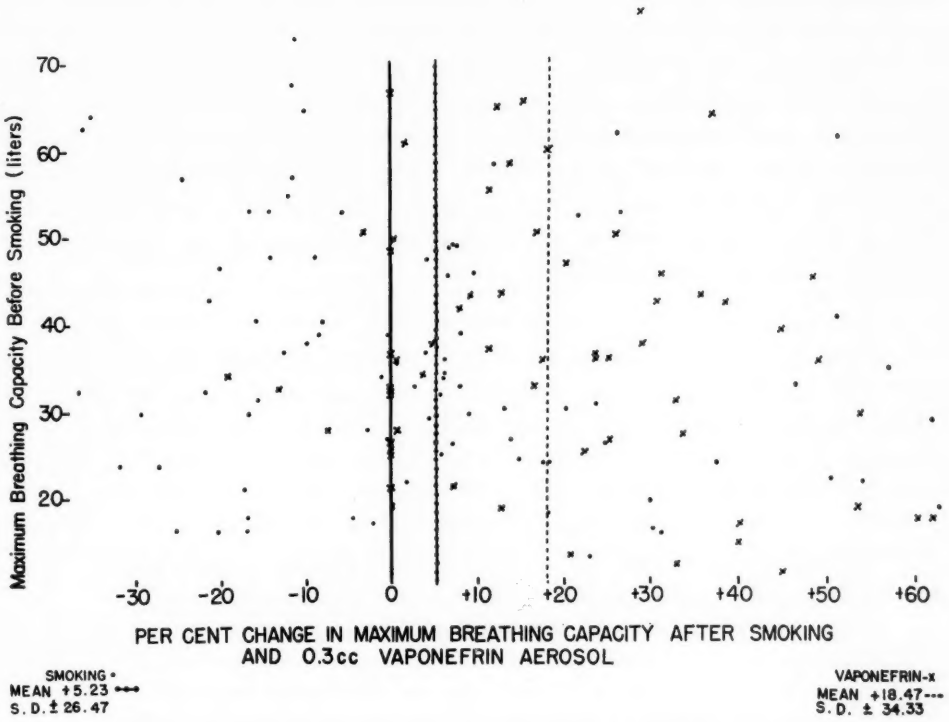

Fig. 2.—Graphic illustration of the per cent change in maximum breathing capacity after smoking and after 0.3 ml. of Vaponefrin aerosol in 91 patients.

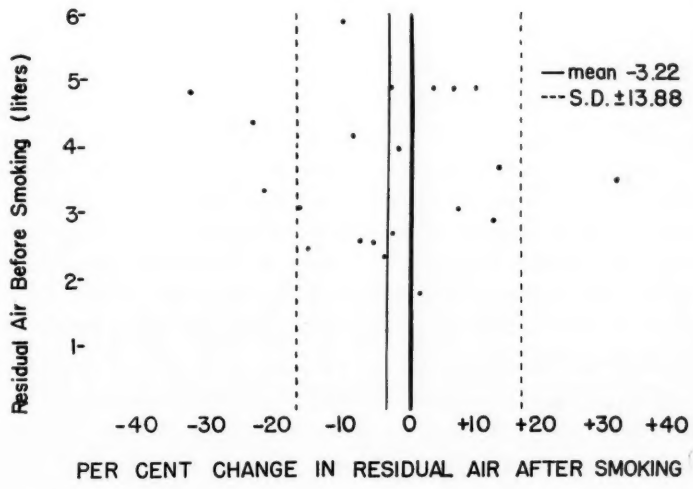

Fig. 3.—The effect of smoking on the residual volume of 21 patients with chronic bronchopulmonary disease expressed as per cent change from the control level.

that these increases in vital capacity, expiratory reserve volume and maximum breathing capacity after Vaponefrin aerosol were due to chance was less than 0.001. This is graphically illustrated in Figs. 1, 2, and 3.

Normal Series.—The mean changes in the ventilatory function tests performed on the normal subjects were slight in magnitude with no significant trend. The mean vital capacity, maximum breathing capacity, and residual volume showed a slight increase after smoking while the expiratory reserve volume, pulmonary ventilation, and ratio of residual volume to total lung capacity fell. The alveolar nitrogen showed an insignificant increase of 0.4 per cent after smoking. The data are presented in Table I.

DISCUSSION

Since the patients and normal individuals in this study were tested before and after smoking three cigarettes, our findings are relevant to the short-term, almost immediate effects of cigarette smoking. Although the majority of the 91 patients tested revealed no noteworthy change in respiratory function, smoking may have increased bronchospasm in the 10 cases in which a reduction in vital capacity and maximum breathing capacity took place. In no instance, however, was a clinically perceptible paroxysm of asthma produced, nor did the patients themselves complain of their subjective states growing worse.

In the 9 patients in whom an increase in vital capacity and maximum breathing capacity was recorded, cigarette smoking was accompanied by a cough productive of mucoid or mucopurulent sputum. Bronchodilator epinephrine compounds, when inhaled as a fine particle-size nebulin, often provoked coughing and expectoration of mucus, with subsequent rise in vital capacity.

The decrease in pulmonary ventilation after smoking in 12 of the 35 patients tested also appeared to be correlated with the expectoration of mucopurulent secretions. The pulmonary ventilation seemed to be diminished, presumably as a result of improved alveolar ventilation. The decrease of the resting minute ventilation when 100 per cent oxygen is substituted for air has been used by the authors as a test of pulmonary function in patients with pulmonary emphysema since the per cent change in ventilation with oxygen becomes less as the degree of dyspnea diminishes.[9, 10] The ventilatory difference became less marked in 9 of the 35 patients tested. Measures that improve respiratory function, such as bronchodilator aerosols which aid the expectoration of retained secretions, also decrease dyspnea with a parallel decrease in pulmonary ventilation breathing air. The acute effects of tobacco smoking did not include, therefore, an increased minute volume of ventilation but a reverse response in some of the patients in whom productive coughing had taken place as a result of the irritant effect of cigarette smoke.

To what extent carbon monoxide in the blood may have altered the pulmonary ventilation is difficult to determine. The study of Asmussen and Chiodi[11] demonstrated that the pulmonary ventilation was not stimulated by CO poisoning as it was in hypoxemia due to breathing a low oxygen atmosphere. In a report by Barach, Eckman, and Molomut,[12] the average carbon

monoxide concentration after inhaling the smoke of 20 cigarettes was 5.7 per cent as compared to 4.0 per cent recorded by McFarland[13] following the inhalation of three cigarettes. Since a reduction in minute volume of ventilation has not been associated with the presence of this blood concentration of CO, the inhalation of cigarette smoke may have resulted in more efficient alveolar ventilation due to the effect of coughing and the resultant elimination of obstructing mucus plugs.

SUMMARY

Respiratory function tests were carried out before and after the smoking of three cigarettes, in a series of 91 patients with bronchial asthma and pulmonary emphysema, and in 27 normal individuals. In 108 of the 118 subjects tested, no evidence of increased bronchospasm or impaired ventilatory function was found, as determined by vital capacity and maximum breathing capacity. Reduction of vital capacity and maximum breathing capacity took place in 10 cases, although unaccompanied by clinically perceptible increase in severity of asthma. An increase of vital capacity and maximum breathing capacity after smoking occurred in 9 patients in whom coughing, provoked by tobacco smoking, resulted in expectoration of mucoid or mucopurulent sputum. A similar but more marked rise in vital capacity was induced by inhalation of bronchodilator aerosols.

In 27 patients measurement of residual volume before and after cigarette smoking showed variations within the range of accuracy of the method. Determination of the minute volume of ventilation, breathing air, and 100 per cent oxygen, apparently revealed no impairment of respiratory function; on the contrary, a decrease in minute volume of ventilation was observed in 12 of 35 tested patients in whom elimination of retained secretions followed the coughing induced by smoking.

The acute experiments reported have no bearing on the effects of habitual smoking on respiratory function.

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LABORATORY METHODS

A RE-EVALUATION OF THE TRYPTOPHAN TEST FOR TUBERCULOUS MENINGITIS

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INTRODUCTION

PROMISING advances of recent years in the therapy of tuberculous meningitis have increased the need for a rapid, simple test for early diagnosis and for prognosis of the disease.

In 1922, Aiello[1] applied a test for tryptophan to spinal fluids and found positive results in each of nine cases of tuberculous meningitis. Five years later, this investigator[2] reviewed the Italian literature on the subject and concluded that out of about a thousand spinal fluid tryptophan tests, 90 per cent of the fluids from patients afflicted with tuberculous meningitis yielded a positive result, and found the incidence of positive reactions in conditions other than tuberculous meningitis not to exceed 2 per cent. Aiello implied that the spinal fluid reaction was given by free tryptophan formed by proteolysis. The test, however, will detect either free tryptophan or tryptophan containing proteins.[3] Mycobacterium tuberculosis does not utilize tryptophan,[4] but tubercle bacilli can synthesize tryptophan.[5] An explanation for the positive reaction in tuberculous meningitis might be nonutilization by the organism of tryptophan released by protelytic processes. Since the tryptophan test is usually positive in general paresis and occasionally in a number of other disorders, the mechanism whereby substances reacting to this test accumulate in the spinal fluid cannot be unique for the tuberculous process.

Lichtenberg[6] was the first to call attention to this test in the American literature, reporting 25 cases of tuberculous meningitis all giving positive reactions for tryptophan in the spinal fluid. Baxter[7] obtained positive tryptophan reactions in 41 cases of tuberculous meningitis, negative results in 31 control cases, and "pseudopositive reactions" in xanthochromic, bloodstained, and purulent cerebrospinal fluids. Other investigators[8-13] were also favorably impressed with the tryptophan reaction. Since positive reactions for tryptophan in neurosyphilis had been noted by previous workers, Madonick and Lissman[14] investigated this phase of the test, and obtained positive reactions in 82 per cent of a series of 133 cases of general paresis.

Recently, Granata and Parenzan[15] in Italy found the tryptophan test positive in only 18 out of 28 cases treated with streptomycin; however, it was not indicated when in the course of the illness these tests were carried out.

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Read before the Feb. 12, 1952, regular meeting of the Chicago Neurological Society.
Received for publication, Sept. 8, 1952.

METHOD

The tryptophan test is simple and is a color reaction. The reagents are: (a) concentrated hydrochloric acid; (b) 2 per cent formaldehyde made from the ordinary 40 per cent formaldehyde (1:20 dilution); (c) 0.06 per cent sodium nitrite solution.

The method we recommend is as follows: Centrifugalize 2 or 3 ml. of cerebrospinal fluid and add to the decanted fluid 10 to 15 ml. of acid and 2 to 3 drops of formaldehyde solution. (If an actual cell count without acidulation reveals a negligible number of erythrocytes, centrifugalization may be omitted.) Shake and allow the mixture to stand for five minutes. Overlay this liquid with 1 to 2 ml. of sodium nitrite solution and allow the material to stand for about two minutes. At least 2 ml. of spinal fluid is necessary, as we have had the experience that with 1 ml. of fluid and 5 ml. of acid the reaction sometimes was negative, although positive if 2 ml. of fluid and 10 ml. of acid were used. At junction of the fluid a delicate violet ring is formed which may take several minutes to become visible and which persists for ten minutes or longer. A negative reaction is characterized by an absence of color change at the junction of the two fluids or by a faint yellowish or brown color. The result was termed pseudopositive in fluids which were grossly bloody, purulent, bloodstained, or xanthochromic. Lichtenberg[6] found the test valid in fluids containing less than 400 red blood cells per cubic millimeter. We observed that in fluids where the blood was removed by allowing it to settle out or by centrifuging and there were considerably more than 400 red blood cells per cubic millimeter, pseudopositive reactions were not given in the decanted fluid if it remained colorless. We have found that if the fluid is not centrifugalized where a red cell count has not been made, or if careful insistence on a true violet color for a positive interpretation is not maintained, false positive reports may result. The test was carried out with ordinary test tubes. An overzealous observer may misinterpret a brownish ring or simply the junctional point of the two fluids as a faint positive reaction. Doubtful observations should be called negative. The readings were made by natural light and artificial light with no difference noted. Viewing the tube against an opal lamp shade over a 100 watt bulb was the practice in our laboratory.

RESULTS

The tryptophan test was performed in 350 cases of patients admitted to Cook County Hospital between May 1, 1950, and Jan. 1, 1952. In every case there was some neurologic or psychiatric symptom or complaint. There were 75 cases of tuberculous meningitis in this series and as a rule several tests were performed during the course of the illness in a given case. The test results in the entire series of cases are shown in Table I.

TABLE I. RESULTS OF TRYPTOPHAN TEST IN 350 CASES

Positive in 92:	74 of 75 cases of tuberculous meningitis
	9 of 11 cases of general paresis
	9 miscellaneous neurologic disorders
"Pseudopositive" in 18:	11 of 30 cases of purulent meningitis
	7 miscellaneous xanthochromic fluids
Negative in 240 cases:	239 control cases, not tuberculous meningitis
	1 case of tuberculous meningitis while under treatment with neomycin

In 74 of 75 cases of tuberculous meningitis (98.7 per cent) the tryptophan test on the spinal fluid was positive at some time during the course of the illness. In 71 cases (94.7 per cent) the first tryptophan test was positive; however, the first test was made on the day of illness varying from the second to the thirtieth day. On the average, the test became positive on the sixth day, varying from

the second to the one hundred and eighteenth day. The latter case is illustrated in Table II. In 89.3 per cent of all the cases the reaction was positive by the sixth day of illness, in 93.3 per cent by the fourteenth day.

The spinal fluid in one necropsy verified case of tuberculous meningitis, in a 13-month-old white girl failed to give a positive tryptophan reaction. Only two tests were made in this case, during the third month of treatment with neomycin. In one other case of this series an initially positive tryptophan reaction became negative under the influence of treatment with neomycin.

TABLE II. TUBERCULOUS MENINGITIS IN A 3-YEAR-OLD NEGRO BOY, TREATED WITH NEOMYCIN FOR SIXTY DAYS, THEREAFTER WITH STREPTOMYCIN AND PROMIZOLE UNTIL DEATH

DAY OF ILLNESS	PANDY	CELLS		GLUCOSE (MG.)	PROTEIN (MG.)	TRYPTOPHAN	REMARKS
3rd day	1 plus	305	38% P. 62% L.	55	56	Not done	Meningeal signs present
61st day	2 plus	75	40% P. 60% L.	72	62	Negative	Clinically well
101st day	Negative	46		—	—	Negative	Clinically well
118th day	4 plus	35	80% P. 20% L.	30	122	Positive	Relapse Culture negative
120th day	—	—		—	—	—	Death

Seven cases of tuberculous meningitis, after a three months' course of dihydrostreptomycin intramuscularly showed change from a positive to a negative tryptophan reaction (see Table III, for an illustrative case).

TABLE III. A CASE OF TUBERCULOUS MENINGITIS TREATED WITH DIHYDROSTREPTOMYCIN FROM APRIL 2 TO JULY 26, 250 MG. INTRAMUSCULARLY EVERY THREE HOURS

DATE	PANDY	CELLS	GLUCOSE	PROTEIN	TRYPTOPHAN	CULTURE
4/ 2	2	346	44	220	Positive	Negative
5/16	2	133	40	95	Positive	Positive
6/13	1	26	54	40	Positive	—
7/26	Negative	6	72	56	Negative	Negative
11/10	Negative	2	60	40	Negative	Negative

Three of these patients at the time of this report were still alive and clinically well. Four suffered relapses and the tryptophan reactions again became positive from one to twenty-seven days after onset of relapse.

We have not encountered any case of tuberculous meningitis which was considered to have been satisfactorily arrested in which the tryptophan test remained positive.

We consider it important to call attention to the poor correlation between the intensity of color reaction and the clinical severity of the meningitis. In most instances the reaction was rated as one plus or a trace, in contrast to the darker rings obtained in xanthochromic fluid or in one of the cases of multiple sclerosis.

The clinical diagnosis of tuberculous meningitis may be attended with considerable difficulty, particularly on admission of the patient to the hospital. For control purposes 275 cases were used, 220 of these representing patients under

the age of 15. Tuberculous meningitis was considered in the differential diagnosis of many of the following 239 cases in which the tryptophan test was carried out on the spinal fluid and found to be negative:

Suppurative meningitis	20 cases
Extraneural tuberculosis, pulmonary, and visceral	20 cases
Epidemic poliomyelitis	15 cases
Upper respiratory tract infection with meningismus	10 cases
Pneumonia with meningismus	10 cases
Status epilepticus	10 cases
Cerebral spastic disease	10 cases
Cerebral thrombosis	10 cases
Miscellaneous conditions	134 cases

As shown in Table IV, 11 purulent and 7 xanthochromic fluids gave so-called false positive reactions. The color was often darker purple than in the case of tuberculous meningitis. The presence of the organism and the fact that purulent fluids are seldom found in tuberculous meningitis are more important than the tryptophan reaction in differentiating these forms of meningitis from the tuberculous variety.

TABLE IV. CASES OF PSEUDOPOSITIVE REACTIONS

NUMBER OF CASES	DIAGNOSIS	TYPE OF FLUID	REMARKS
11	Purulent meningitis, including influenzal and pneumococcic	Grossly purulent	6 recovered 5 necropsies
2	Subacute bacterial endocarditis	Xanthochromic	Survived
1	Astrocytoma of medulla	Xanthochromic	Necropsy
1	Mumps meningo-encephalitis	Xanthochromic with 600 RBC/cu. mm.	Recovered
2	Pulmonary tuberculosis	Xanthochromic	Later developed clinical tuberculous meningitis with positive reaction on colorless fluid
1	Guillain-Barré syndrome	Xanthochromic	Improved. Spinal protein over 1,000 mg./100 c.c.

Eighteen of the remaining cases of disease conditions other than tuberculous meningitis in this series were marked by positive reactions. Nine were cases of general paresis (with typical spinal fluid formula), and three were cases of multiple sclerosis with a first-zone Lange reaction. In six other cases a clear fluid had not been centrifugalized and yielded a trace reaction. These included two cases of cerebral hemorrhage, and one each of epidemic poliomyelitis, progressive muscular atrophy, muscular dystrophy, and epilepsy.

DISCUSSION

Some years ago the senior author, on becoming acquainted with Lichtenberg's study was impressed by the simplicity of this test tube reaction, and used it as an adjunctive diagnostic measure in cases of suspected tuberculous meningitis encountered in his private practice. The test proved of particular value in consultation practice, where in a matter of minutes after fluid was secured by spinal puncture, a negative tryptophan reaction constituted a sort of presumptive exclusion test when tuberculous meningitis was suspected. Then in 1945 a case of perfectly typical tuberculous meningitis, with otherwise

classical spinal fluid findings was encountered in which the test was negative in each of five spinal fluid examinations during the course of this fatal illness. From the present series of cases of tuberculous meningitis one might secure the impression that the test would have been found positive in every untreated case at some time during the course of the illness, but the author's personal experience just mentioned refutes this.

It is generally true that on the spinal fluid findings alone can tuberculous meningitis be differentiated from certain disorders simulating it which fall into three general groups. These include: (1) primary neurotropic virus diseases, such as lymphocytic choriomeningitis, and the several forms of acute epidemic encephalitis; (2) the occasional meningo-encephalitic inroads of such primary viscerotropic virus diseases as mumps, infectious mononucleosis, and so forth; and (3) aseptic meningeal reaction, secondary, for example, to deep-seated brain abscess. In all these disturbances there may be predominantly lymphocytic pleocytosis with a negative Wassermann test and failure to isolate a causative microorganism on the usual cultural media. The differential criteria are ordinarily the sugar and chloride content of the fluid, and even more so a certain combination of findings in the spinal fluid which Merritt and Fremont-Smith[16] have emphasized is highly indicative of tuberculous meningitis. Yet in practice, this differentiation is not so easy because of (1) technical and laboratory errors, and (2) failure of one or more of these criteria in individual cases or in fluids obtained at different times during the course of the illness. In two of our cases of tuberculous meningitis, the sugar content of the spinal fluid was normal or elevated on several occasions; and in a third case, the only spinal fluid specimen obtained, a week before death, revealed 200 cells per cubic millimeter, 50 mg. of glucose and 122 mg. of protein per 100 ml., 115 meq. of chloride, and a positive tryptophan reaction. In all three cases there was necropsy verification of the diagnosis.

We found positive tryptophan reactions in 9 of 11 cases of paresis, in agreement with the findings of Madonick and Lissman,[14] but here the Wassermann reaction on the fluid was strongly positive. The occasional positive reactions in miscellaneous neurological conditions such as in some cases of multiple sclerosis will ordinarily not cause any confusion since the clinical picture and other spinal fluid characteristics are incompatible with the diagnosis of tuberculous meningitis. In this respect the test is nonspecific, somewhat akin to a first zone Lange reaction, which is occasionally encountered in brain tumor and divers other central nervous system disorders besides general paresis and multiple sclerosis, and cannot be evaluated by itself without respect to other spinal fluid constituents and clinical study of the patient.

SUMMARY

The technique of the tryptophan test on the spinal fluid has been outlined, with the provision that the specimen first be centrifuged, as otherwise contaminating red blood cells might give a false positive reaction; and the occurrence of so-called pseudopositive reactions with xanthochromatic and grossly purulent fluids have been stressed.

Positive tryptophan reactions in relatively clear colorless fluids were obtained in 89 per cent of 75 cases of tuberculous meningitis by the sixth day of the disease, and in over 98 per cent of cases at some time during the course of the illness.

With clinical arrest of the disease process under the influence of antibiotic therapy the reaction becomes negative.

Positive reactions were obtained in general paresis, and in a few miscellaneous other conditions usually unassociated with pleocytosis.

The tryptophan reaction when positive, and with a negative Wassermann test in a colorless, relatively clear cerebrospinal fluid showing pleocytosis is strong presumptive evidence of tuberculous meningitis. The test, however, is in no sense pathognomonic; and a negative reaction under the conditions set forth does not exclude the diagnosis of tuberculous meningitis.

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THE DETERMINATION OF PYRAMIN

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PYRAMIN is a pyrimidine metabolic breakdown product of thiamine which is excreted in urine. It is determined by a yeast fermentation method[1] after the thiamine has been destroyed by sulfiting.[2] The values for it are reported in terms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride.

Mickelsen, Caster, and Keys,[3] from a long-term study of normal young men maintained on controlled thiamine intakes, report that pyramin excretion may serve as a useful index of thiamine metabolism. They describe the relation between thiamine intake and pyramin excretion in quantitative terms and present data defining normal excretion levels. It had previously been stated by Pollack, Ellenberg, and Dolger[4] that whereas thiamine excretion reflected only the immediately preceding vitamin intake, pyramin excretion more faithfully reflected the body stores of thiamine. Similar conclusions can be drawn from the data of Wertz and Mitchell.[5] Gorham, Abels, Robins, and Rhoads[6] also present evidence which indicates that, following a constant dose of thiamine, the excretion of pyramin shows less interindividual variation than does the excretion of thiamine.

This paper describes the conditions of the fermentation procedure which we have found most satisfactory for the determination of pyramin and discusses factors influencing the interpretation of pyramin excretion figures.

MATERIALS AND METHODS

Reagents.—Sulfiting solution—The reagents used are those described by Schultz, Atkin, and Frey.[1]

Pyrimidine standards—A solution of 100 γ of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride per milliliter is made up in 0.01 N HCl and stored in a refrigerator. This stock solution was found to be stable for several years. This stock solution is diluted, as needed, to a concentration of 0.1 γ per milliliter with distilled water and this dilute solution is used directly as a standard in the fermentation. Routinely, six standards containing 0, 2, 4, 6, 8, and 10 ml., respectively, of this dilute solution are used as standards in each fermentation run.

Yeast suspension—25.0 Gm. of Fleischmann's Bakers' Yeast are added to 500 ml. of distilled water in a 600 ml. beaker. Constant, vigorous mechanical stirring is maintained during the time that the yeast suspension is being pipetted, and for at least five minutes prior to that. Calibrated Krogh-Keys syringe pipettes[7] are used to measure this suspension

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This work was supported in part under the terms of a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Regents of the University. Financial assistance was also provided by the Sugar Research Foundation, Inc., New York, and the National Dairy Council, operating on behalf of the American Dairy Association, Chicago. Merck & Co., Inc., Rahway, N. J., provided the crystalline compounds.

Received for publication, Oct. 15, 1953.

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into the fermentation flasks as a means of both increasing the accuracy and greatly decreasing the time required at this stage.

Nutrient solutions—The nutrient solutions used in the fermentation are those of Schultz and co-workers[1] to which pyridoxine[8] has been added. Solution A consisted of: 180 Gm. $NH_4H_2PO_4$, 72 Gm. $(NH_4)_2HPO_4$, 0.2 Gm. nicotinic acid, and 4 mg. of pyridoxine made to 1 liter with distilled water. Solution B contained 200 Gm. anhydrous dextrose, 7.0 Gm. $MgSO_4 \cdot 7H_2O$, 2.2 Gm. KH_2PO_4, 1.7 g. KCl, 0.5 Gm. $CaCl_2 \cdot 2H_2O$, 10 mg. $FeCl_3 \cdot 6H_2O$, and 10 mg. $MnSO_4 \cdot 4H_2O$ made to 1 liter with distilled water. Two or three liters of each solution are prepared at one time and sterilized in 4-liter Pyrex bottles equipped with syphon delivery tubes. These storage bottles are so arranged that the air entering the bottle passes through a $CaCl_2$-tube filled with sterile cotton. The tip of the delivery tube on the end of the syphon is covered with a test tube except when actually in use. Sterilization is accomplished by autoclaving at 100° C. for forty-five minutes on each of three successive days. It was found that stock solution prepared in this way could be used intermittently over periods of as long as six months with no detectable deterioration or signs of bacterial growth. Krogh-Keys syringe pipettes[7] are used to add aliquots of these solutions to the fermentation vessels.

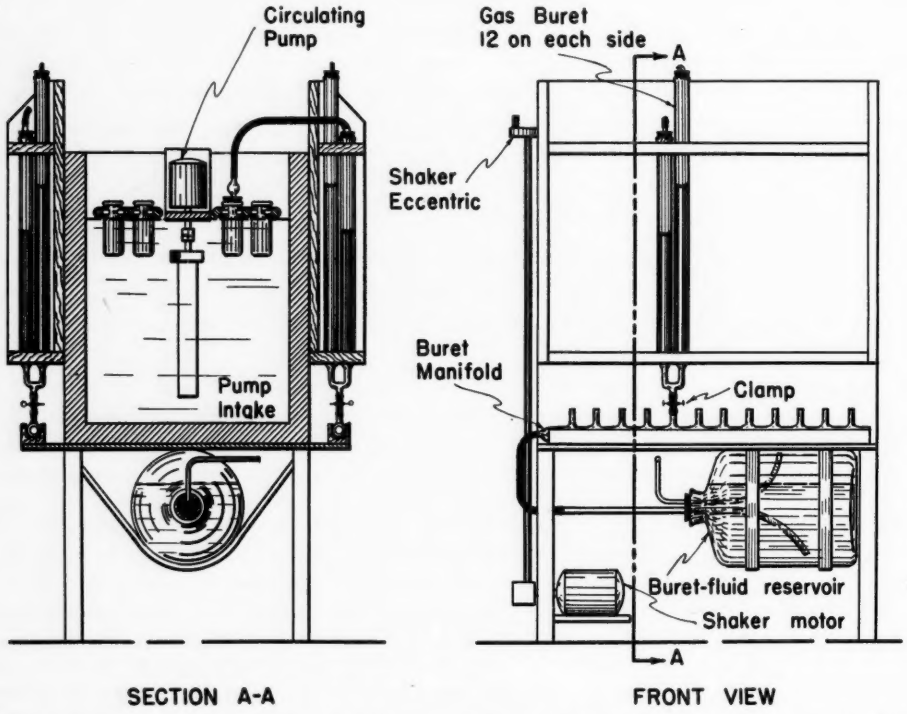

Fig. 1.—Fermentometer. Section *A-A* is a cross-section taken at the line *A-A* on the front view.

Fermentometer.—The fermentometer used in this work is similar to the commercially available model except that it has a larger capacity and more exact controls. The details of its construction are shown in Fig. 1. The shaking rate is 138 excursions per minute.

By replacing the bulky and clumsy leveling bulbs with "leveling tubes" of approximately the same diameter as the burettes, it was found possible to (a) enclose the gas burettes within an air bath to help maintain conditions of constant temperature, and (b) fit 12 complete burette-leveling tube pairs along a 39-inch side of a water bath. The burettes are of 250 ml.

capacity, graduated at each milliliter. Equalizing the liquid levels is accomplished by pressing the pinch clamp at the base of the Y-tube connecting the burette-leveling tube pair. This allows excess liquid to flow down into the large reservoir bottle. At the end of the fermentation the direction of flow is reversed by applying air pressure to the reservoir bottle and again releasing the pinch clamp.

The fluid used in the system is distilled water to which 3 Gm. per liter of $ZnCl_2$ and 1 ml. per liter of 85 per cent H_3PO_4 have been added to inhibit bacterial growth and to give an acidity below pH 4. Reading the meniscus is facilitated by (a) a long fluorescent light set just outside of the bottom of the air bath, and (b) ''meniscus markers'' placed on the surface of the liquid within the burette. These meniscus markers are made by pouring paraffin, colored with Sudan IV, into the depressions of a spot plate. The bottom of this artificial red meniscus furnishes a stable reference point that can be easily read even when the liquid surface is disturbed by the vibration of the shaking mechanism. With this equipment, readings can be made without interrupting the fermentation in any way.

Preparation of Samples.—Twenty-four-hour samples of human urine are collected with 3 ml. of toluene and 5 ml. of glacial acetic acid as preservative. Each urine sample is well mixed, measured, and a 30-ml. aliquot pipetted into a test tube which has been calibrated at 35 and 50 ml. (Folin-Wu N.P.N. tube).

One milliliter of 15 per cent Na_2SO_3 and 3 drops of 9N NaOH are added to each tube. The samples are mixed by closing each tube with a rubber stopper and inverting the whole rack of tubes several times. Using bromcresol purple as an outside indicator all tubes are adjusted to approximately pH 5.5. Each tube is covered with a small beaker, and the rack is placed in a bath of boiling water. A small laboratory towel is placed over the top as a loose cover. Thirty minutes after the bath comes to boil, the rack is removed and immediately 1.1 ml. of 3 per cent H_2O_2 is added to each tube with shaking. When the samples cool to room temperature, the rack is placed in a refrigerator overnight.

The following day the samples are shaken, tested for excess sulfite, and adjusted to pH 7.0 using bromthymol blue as an outside indicator. This requires approximately 3 drops of 9 N NaOH and a final adjustment with 1 N acid or alkali. The starch-iodide indicator used to check the sulfite end point is the same as that used by Schultz, Atkin, and Frey[1] with the possible exception that the reagents are invariably mixed in the following order: 1 drop of 50 per cent H_2SO_4, 1 drop of 5 per cent KI, and then 1 drop of 1 per cent starch. On standing a few moments this indicator develops a pale rose color which becomes dark blue with the addition of peroxide and is decolorized in the presence of sulfite. It is usually found that the sulfited sample either produces no change in the color of the indicator spot or causes it to fade slowly. If the color fades completely in less than ten seconds, further adjustment is made with 3 per cent peroxide.

The volume of liquid in the tubes is adjusted to 50 ml. with distilled water. Occasionally when the pyramin excretion is expected to be exceedingly low, the samples are made up to the 35 ml. mark. Samples are sulfited within three days after the urine was collected. The sulfited samples are stored in the refrigerator for as long as two weeks. After twenty to thirty days' storage, much lower values are occasionally observed. Samples are routinely run in duplicate with the two aliquots usually being of different sizes.

Fermentation Procedure.—In each of the 24 fermentation vessels place (a) an appropriate aliquot of sulfited urine sample or standard, (b) sufficient distilled water to bring the liquid volume to 30 ml., (c) 2.5 ml. of Solution A, and (d) 7.5 ml. of Solution B. The bottles are then clamped into the metal frame and placed in the fermentometer. The addition of yeast suspension to successive vessels is made at exactly ten-second intervals marked by an automatic time signal. With two people working together at this point the yeast was added to all 24 fermentation vessels, the stoppers fastened into position, and the shaking started with $3\frac{1}{2} \pm \frac{1}{4}$ minutes after the ''zero time.'' The zero time of the fermentation is taken as the time the yeast suspension is added to the first fermentation vessel. The initial gas volume readings are made twenty minutes later without interrupting the shaking. The final readings are made in similar fashion exactly three hours after the zero time. In both cases the readings are recorded on successive tubes at ten-second intervals as marked by the time signal.

In the calculation of results all gas evolution volumes are corrected for temperature, pressure, and the vapor pressure of water. A standard curve is constructed by plotting the gas evolution above the blank observed for each standard against the micrograms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride present in that standard. The standard curve used in this work is seen in Fig. 2. This standard curve was found to be highly repeatable from day to day over the course of several years. Urinary pyramin excretion values are calculated by reference to this standard curve and are reported in terms of micrograms of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride.

Precision.—With standards, a repeatability of $\sigma = 1.1$ ml. of CO_2, or roughly 3 per cent relative analytical error, can be attained with this equipment. In routine work with sulfited urine samples a deviation of $\sigma = 1.5$ to 1.8 ml. CO_2 is more usual. This amounts to a 5 to 6 per cent relative error in pyramin values; or an error of perhaps 10 γ of pyramin per day in urinary excretion level. Factors affecting this total method error have been analyzed statistically further.[10]

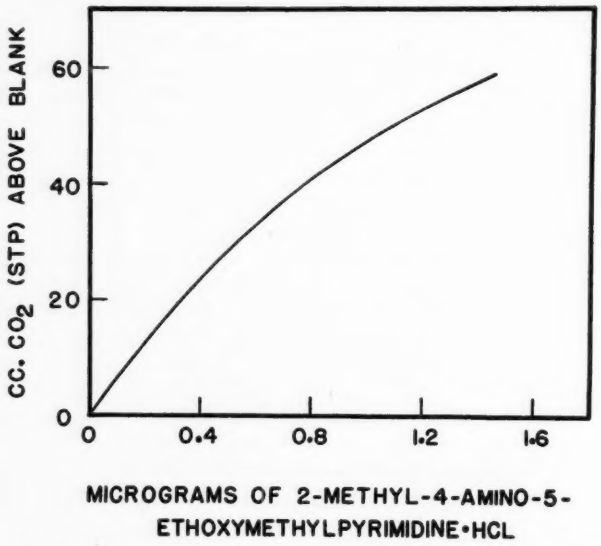

Fig. 2.—Standard curve used for calculating pyramin values.

RESULTS

Utility of Results.—Mickelsen, Caster, and Keys[3] describe the relation between thiamine intake and pyramin excretion in quantitative terms and present estimates of the variation associated with these values. At an average intake of 1 mg. of thiamine per day, for example, an average pyramin excretion of about 170 γ/24 hours was observed. The pyramin excretion level increased more slowly at higher thiamine intakes. From 1 to 2 mg. of thiamine intake per day, the increase was only 70 γ pyramin per change of 1 mg. of thiamine intake, while between the intakes of 0.5 to 1.0 mg. of thiamine, the pyramin levels differed by 130 γ pyramin per milligram of thiamine intake.

At the same time there was a random variation of $\sigma = 20.8$ γ associated with each pyramin excretion value, and an additional individual variation of

$\sigma = 8.8\ \gamma$ between the long-time means of different men on the same intake of thiamine. Taken together, these variations result in an uncertainty of $\sigma = 22.6\ \gamma$ associated with each pyramin value.

This 22.6 γ is a fairly large variation in terms of the difference of 70 γ pyramin/mg. of thiamine intake encountered at high thiamine intakes, but quite acceptable when compared with the difference of 130 γ pyramin/mg. of thiamine intake found at lower levels of thiamine intake. In other words, pyramin is a particularly useful measure for studying low levels of thiamine intake.

Only one factor has been discovered which interferes with the utility of pyramin values, and this is the presence of heat-degradation products of thiamine in the diet. Several of the experimental diets used in the work reported by Mickelsen, Caster, and Keys[3] contained meats which had received an unusual amount of heat treatment and were thus low in thiamine content. This may account for much of the day-to-day variation of $\sigma = 22.2\ \gamma$ of pyramin noted by these workers.[3] Diets used during acute deficiency periods[11] were supplemented with autoclaved yeast extract. Unusable pyramin values resulted. Caster, Mickelsen, and Keys[9] have discussed this effect. In rat experiments using a diet containing autoclaved peanuts, Caster[12] has also noted grossly abnormal pyramin values.

Autoclaved products of this type are not consumed in large amounts in ordinary human diets, but where they do occur pyramin values can be expected to be correspondingly high.

SUMMARY

A modification of the yeast fermentation method for the determination of pyramin (the pyrimidine degradation product of thiamine) excreted in the urine is presented which increases the accuracy and reproducibility of the method.

The utility of the urinary pyramin values as an index of thiamine metabolism is presented.

We wish to thank Mr. Eugene Sunnen and Mr. Ersal Kindel for their aid in constructing the fermentometer.

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DETERMINATION OF INULIN IN PLASMA AND URINE BY USE OF ANTHRONE

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INTRODUCTION

THE wide use of inulin as a biologic tool emphasizes the importance of a simple method for its determination. Present methods depend upon the color development of hydrolyzed inulin with resorcinol or diphenylamine[1-4] reagent.[5-9] This paper describes a new method for the determination of inulin based on an adaptation of the color reaction between polysaccharides and anthrone reported by Dreywood.[10] It has certain advantages over the previous methods and has excellent reproducibility.

METHODS AND MATERIALS

Reagents.—

A. Anthrone Reagent: Dissolve 200 mg. of anthrone (Eastman Kodak) in 100 ml. of concentrated sulfuric acid. This reagent should be prepared on each day of use.

B. Twenty-five per cent trichloracetic acid: Dissolve 25 Gm. of trichloracetic acid crystals in distilled water and dilute to 100 ml.

C. Yeast Suspension: Approximately 20 per cent suspension is made from baker's starch-free yeast (Standard Brands, Inc.). The yeast is washed and centrifuged several times with distilled water until the supernatant fluid is *clear*. After the last centrifugation, the suspension is made up to approximately 20 per cent by volume with distilled water. This yeast suspension may be kept by refrigeration for several days, washed again on each day of use, and made up to the original volume.

Procedure.—

A. Plasma: To exactly 1 ml. of oxalated plasma in a centrifuge tube, add 3 ml. of a 20 per cent suspension of baker's starch-free yeast. Incubate at room temperature for 15 or 20 minutes with occasional shaking. Centrifuge the yeast down for 10 minutes at about 1,800 r.p.m. Decant the supernatant fluid quantitatively into a centrifuge tube and treat the supernatant with 1 ml. of 25 per cent trichloracetic acid. Mix thoroughly and let stand for about a minute. Wash the inside of the tube with approximately 0.5 ml. of distilled water and then centrifuge out (15 minutes at 1,800 r.p.m.) the resulting plasma protein precipitate.

Carefully pour off the supernatant fluid from the centrifuge tube into a calibrated test tube and bring the total volume to exactly 6 ml. with distilled water. Transfer 4 ml. of this supernatant into a 25 by 200 mm. Pyrex tube (this size is critical for optimum color development[11]). Add 8 ml. of anthrone reagent while swirling the tube vigorously. Cool in air to room temperature. The samples are then read in a spectrophotometer at 6,200 Å.

A standard curve is prepared by adding known quantities of inulin to samples of control plasma. This curve should be prepared with each determination. The inulin concentration of the experimental samples is calculated from the standard curve.

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Received for publication, Oct. 27, 1953.

B. Urine: The expected urine inulin concentration is diluted so that its ratio to that of the plasma is approximately 1.0. After dilution, the urine is treated in the same manner as described for plasma.

A standard curve is prepared by adding known quantities of inulin to samples of diluted control urine (about 1 to 50 parts water). This curve should be prepared on the same day that the unknowns are determined. The inulin concentration of the experimental samples is calculated from this curve. Precipitation with trichloracetic acid and yeasting is not necessary if the urine is known to be free of protein and glucose.

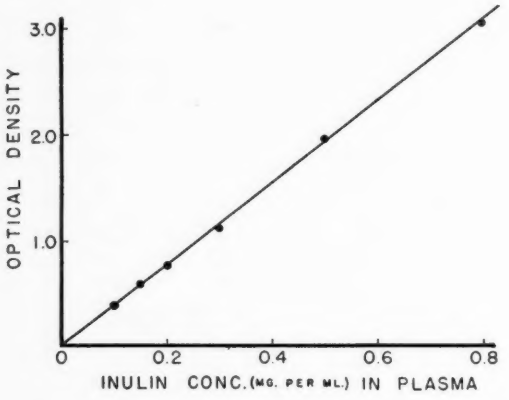

Fig. 1.—Recovery of inulin added to plasma. Concentration represents the amount of inulin in the original one milliliter sample analyzed. The optical density was measured with a Beckman Model B Spectrophotometer.

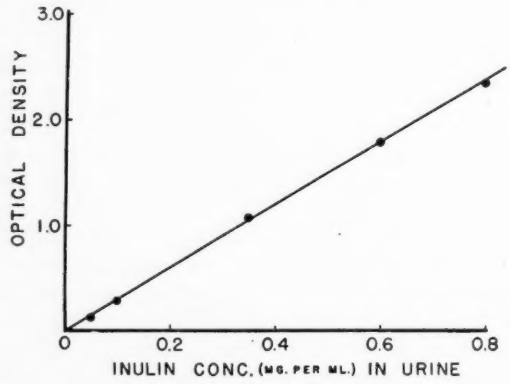

Fig. 2.—Recovery of inulin added to diluted urine. Concentration represents the amount of inulin in the original one milliliter sample analyzed. The optical density was measured with a Beckman Model B Spectrophotometer.

RESULTS AND DISCUSSION

The color reaction of polysaccharides to anthrone reagent described by Dreywood[10] may be modified for use in determining inulin. The results of recovery experiments in which known amounts of inulin were added to plasma

are shown in Fig. 1. The results of a similar recovery of inulin added to urine are presented in Fig. 2. It is clear from these results that the method is reliable for the analysis of inulin in plasma and urine.

The color developed with anthrone not only depends upon the concentration of inulin, but also upon the degree of heating. Therefore, the reaction tube size is critical and the rate of addition of the anthrone reagent should be uniform. Furthermore, it is important that all the samples are cooled in the same manner (e.g., standing in air) since the rate of cooling will influence the final color development. It was found that rapid cooling will alter the optical density readings at 6,200 Å by a factor of three or more; the samples which are immediately cooled give a higher optical density reading than those cooled at longer intervals. Seifter and co-workers[12] describe a method of controlled heating which does not require a particular tube size. In our experience, however, this technique offers no advantages over the procedure presented previously and is time consuming.

Since the anthrone reagent deteriorates on standing, it is preferable to prepare a standard inulin curve at the time the unknowns are determined.[11, 12] The final green color developed is stable for many hours and has a maximum light absorption at 6,200 Å.

Contamination by polysaccharide material such as pieces of cotton or bits of filter paper must be avoided. In this respect the use of cellulose nitrate centrifuge tubes is unsatisfactory.

Although some of the recent methods attempt to circumvent yeasting,[2, 9] in the opinion of the authors yeasting remains the most satisfactory method for avoiding the chromogenic interference of glucose.

SUMMARY

A new, rapid photometric method for the determination of inulin in plasma and urine based upon the color reaction between anthrone reagent and inulin is presented. This method is simpler and faster than the older methods and is practical for use in clinical and research laboratories.

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A SIMPLIFIED ALTMANN-GERSH FREEZING-DRYING APPARATUS

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IN 1932 Gersh[1] introduced a method for the fixation of tissue for microscopic study by the "freezing-drying" technique. This method was developed on the principles suggested by Altmann[2] in 1890. In using this technique, tissue is frozen immediately upon removal from the animal. It is then placed in a refrigerated vessel, and dehydrated at low pressure while still in a frozen state. This procedure has distinct advantages over other methods of fixation in that it causes little, if any, denaturation of proteins, reduces diffusion of cellular components to a minimum, and practically eliminates post-mortem changes in the tissues so treated. In many respects this is an ideal method not only for the preservation of cellular detail, but also for histochemical analysis of tissue.

The apparatus and technique now generally used in this method are, with minor modifications,[3-6] essentially the same as that initially described by Gersh.[1] This apparatus has the limitations of being both cumbersome and expensive. Without these restrictions, this method could have a wider application and more general use in pathology, cytology, and histochemistry. An apparatus of simple design and little expense embracing the principles enunciated by Altmann,[2] Gersh,[1] and Hoerr[3] has been constructed in this laboratory. For the past two years it has produced results consistently comparable to those of the more complex apparatus.

APPARATUS

The accompanying photograph (Fig. 1) illustrates the general appearance of the apparatus. A diagrammatic sketch (Fig. 2) presents constructional features of the insulated box which was built of ¼ inch plywood and ¾ inch supporting lumber. A one inch space (*A*) between the outer box (*1*) and the inner chamber (*2*) was packed with rock wool as insulation. The Dry Ice used to obtain the cold temperatures required is placed in compartment *B*. This compartment is separated from the freezing compartment (*C*) by a plywood partition (*3*). By increasing the thickness and insulating properties of this partition, the temperature in the freezing compartment (*C*), where the specimens are placed in the glass container, may be adjusted. The glass apparatus† consists of a refrigerated chamber (*4*), in which the tissues are placed in a small wire basket (*5*). This chamber is kept at a low temperature so that the tissues remain in a frozen state during dehydration. Connected to the refrigerating chamber (*4*) is a drying chamber (*7*) which houses a removable glass vessel (*8*) that contains phosphorous pentoxide as the dehydrating agent. The glass tube connecting the refrigerating and the drying chambers passes through a slot in the insulated box

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Received for publication, Nov. 4, 1953.

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†The glass apparatus was made to our specification by Mr. C. C. Van Hespen, University of Chicago, Chicago, Ill.

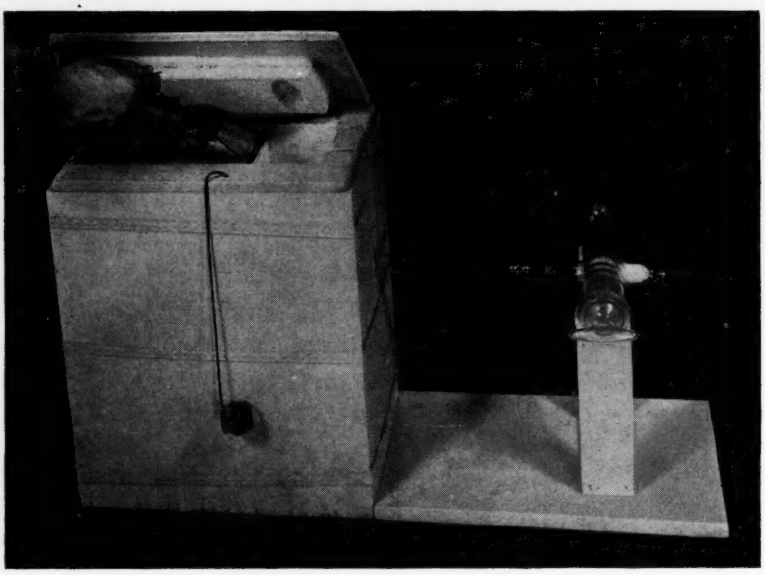

Fig. 1.—Assembled freezing-drying apparatus.

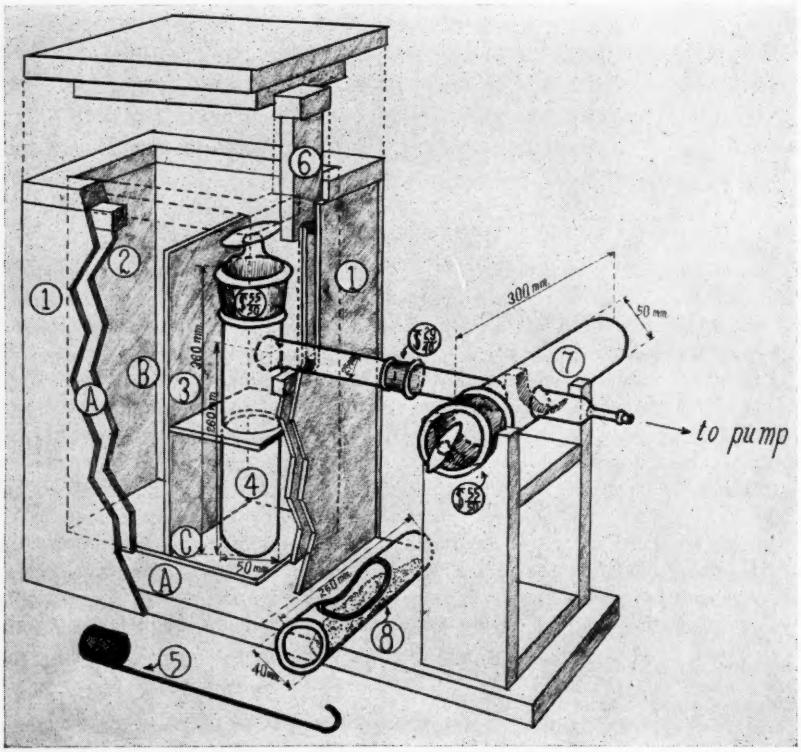

Fig. 2.—Diagrammatic sketch of constructional features of the freezing-drying apparatus.

which is closed during operation by a sliding partition (*6*). The glass apparatus is connected to an efficient high vacuum rotary oil pump capable of maintaining a pressure of 10^{-3} mm. of mercury. A single charge of Dry Ice of approximately 25 pounds will maintain a temperature of -30° C., at the bottom of the glass refrigerating chamber (*4*), for over twenty-four hours.

In this procedure the fresh tissue is placed on a small piece of paper with proper identification and is immediately frozen in isopentane cooled to about –155° C. with liquid nitrogen. The tissue adheres readily to the paper throughout the freezing and drying process. This frozen tissue is placed in a wire basket and transferred to the glass refrigerated vessel where it is dried under vacuum at a temperature of about -30° C. Drying is complete when the phosphorus pentoxide no longer accumulates any moisture on its surface. Two to three pieces of tissue, each measuring 10 mm. by 20 mm. by 1 mm., are dried in twelve hours in this apparatus. Thin pieces of tisue are essential for complete drying. Additional pieces of the same size prolong the time thirty-six to forty-eight hours or longer.

When drying is completed the tissue is removed from the apparatus and dropped into a tube of melted paraffin (50° to 52° C.). The tube is connected to a vacuum pump and maintained at a pressure of about 10^{-2} mm. of mercury for ten minutes. The paraffin is kept liquid by the occasional application of heat by a Bunsen burner to the outside of the tube. In about ten minutes the infiltration tube is returned to atmospheric pressure. This completes the process of infiltration. The tissue is then blocked in paraffin in the conventional manner.

Tissues processed in this simplified apparatus are comparable in structure and physical properties with similar material prepared in the conventional Altmann-Gersh apparatus. For example, the previously described[7] solubility properties of the cytoplasmic glycoprotein component of the dog prostate were identical in tissue prepared in this simplified apparatus. Similarly, the distribution of glycogen in the "ovoid granular cell" of the prostate was the same in both techniques.

From these and other observations on relatively labile substances in tissues, we believe that our histologic preparations compare favorably with those obtained with the conventional, more complex apparatus.

SUMMARY

A simplified, inexpensive modification of the Altmann-Gersh freezing-drying apparatus is presented. The results obtained from its use are comparable to those of the more complicated apparatus.

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A SIMPLE INDIRECT METHOD FOR DETERMINING PLASMA SODIUM LEVELS

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WITH the recent advances in flame photometry[1] and its widespread use in medical research has come increased understanding and awareness of the role played by electrolyte metabolism in both health and disease. This has brought forth a large body of knowledge concerning the diagnosis and treatment of the various electrolyte disturbances. Because of the expense, however, the necessary laboratory facilities and the trained personnel required for its operation, the flame photometer determinations of sodium and potassium are not available to a large segment of the medical profession who practice away from the larger medical centers.

Where the flame photometer is not available, the electrocardiogram together with other diagnostic criteria available can provide some indication of plasma potassium levels. At times, the level of plasma sodium can be approximated by adding the carbon dioxide combining power in milliosmols per liter and chlorides in milliequivalents per liter, plus an additional factor to allow for the other anions such as phosphate, etc.[2] Unfortunately, where serious electrolyte imbalances are present and fluid and electrolyte therapy is indicated, the above approximation does not hold true.

For many years the possibility has been considered as to whether the erythrocyte acts as a perfect osmometer; i.e., adjusts itself to changes in osmotic pressure by the shifting of water alone. Results have varied.[3] Guest and Wing[4] found that the swelling of normal human erythrocytes suspended in a series of hypotonic salt solutions followed closely that expected if they behaved as perfect osmometers. Guest[5] did similar studies using abnormal erythrocytes from patients with hypochromic microcytic anemia, pernicious anemia, and sicklemia, etc., and found that these cells did not act as perfect osmometers, but from his data one can conclude that the relationship between cell volume and tonicity of the external medium is essentially linear within the range of tonicities with which the method in this paper is concerned.

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This work was supported by grants from the United States Public Health Service and United States Army Contract No. DA-49-007-MD-465.

Received for publication, Nov. 5, 1953.

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The permeability of the erythrocyte to various ions has been studied by many investigators. Anions such as chloride and bicarbonate appear to penetrate the cell rapidly.[6] Wakesman and his associates[7] thought from their studies that the red cell was impermeable to sodium and potassium and that equilibrium was re-established by the transfer of water, carbon dioxide, and chloride between cells and plasma. Eisenman and his co-workers[8] gave intravenous infusions of hypertonic saline to patients who were suffering from severe dehydration and plasma base deficiency. They found changes in cell volume shortly after the infusions and interpreted these to mean that the erythrocyte in the circulating blood acted as if it were impermeable to base. Chemical analysis, on the other hand, showed that significant quantities of base seemed to cross the cell membrane, and they explained this paradox by stating that this shift of base was probably due to metabolic activities rather than to osmotic adjustment. The theory of apparent impermeability of the erythrocyte to cations has apparently been shattered by isotope studies[9, 10] which show that erythrocyte potassium and plasma potassium exchange at a uniform rate, the exchange rates found being approximately 1.6 to 1.8 per cent of the cellular potassium per hour.

Nevertheless, in spite of these studies, it is known that great ionic concentration gradients of sodium and potassium exist between the erythrocyte and the plasma. Also, water apparently moves rather freely between these two compartments in response to osmotic changes and in a direction which produces osmotic equilibrium.

Before the turn of the last century, Gryns,[11] as well as other workers, noted this relationship. Gryns[11] devised a procedure by which he determined the osmotic activity of the serum based on changes in erythrocyte volume. Warburg and Winge[12] developed a method for determining relative total base concentration of the serum employing the principles of this earlier work. Although later modified,[13] the method remains tedious and requires special equipment.

Since sodium makes up most of the base of the extracellular fluid, it controls almost entirely the osmotic stability of this medium. Therefore, it was decided to investigate the relationship between erythrocyte volume and plasma sodium concentration. From this work a simple indirect method for determining plasma sodium was derived. The procedure is such that it can be carried out with the facilities usually available in the routine laboratory.

The individuals selected for this study were patients on the medical and surgical services of Grady Memorial Hospital and Atlanta Veterans Administration Hospital. Most of these patients were suspected of having electrolyte disturbances. The following were among the disease states included: Addison's disease, diabetes mellitus, malignant hypertension, uremia, both chronic and acute, postoperative states, intestinal obstruction, Laennec's cirrhosis, chronic lung disease with respiratory acidosis, congestive heart failure, etc.

MATERIALS AND METHODS

Materials.—

a. Four 4 ml. hematocrit tubes* with rubber sleeve stoppers.†

b. Three 5 ml. volumetric pipettes (used for filling hematocrit tubes and adding solutions).

c. One long dropper (for removing plasma).

d. International centrifuge Size 1 with Head No. 240 (or similar centrifuge).

e. Blood bottle with rubber stopper in which 1 ml. of heparin solution containing 3 mg. of heparin sodium is allowed to dry at room temperature. The heparin solution is made up by diluting 3 ml. of sodium heparin‡ (10,000 units per milliliter) to 100 ml. with distilled water.

Solutions.—

I. Solution A (hypertonic) 155 meq./L. of Na as NaCl plus 6 meq./L. of K as KCl.
9.0604 Gm. NaCl and 0.4473 Gm. KCl are weighed out and diluted up to one liter with distilled water.

II. Solution B (hypotonic) 105 meq./L. of Na as NaCl plus 6 meq./L. of K as KCl.
6.1377 Gm. NaCl and 0.4473 Gm. KCl are weighed out and diluted up to one liter with distilled water.
The above compounds are C.P. reagents which are dried at 110° C. for twenty-four hours. Solutions are stored in glass-stoppered Pyrex bottles which are kept tightly stoppered and shaken well before using.

Method.—With the aid of a tourniquet, 20 ml. of venous blood are drawn and placed in a heparinized blood bottle which is then tightly stoppered. Within two hours after drawing the blood, the following procedure, which includes duplicate determinations, is carried out. The blood is carefully mixed to obtain even distribution of the red cells. Four 4 ml. hematocrit tubes are filled to the 4 ml. mark, capped tightly and centrifuged at 3,000 r.p.m. for thirty minutes. Then the hematocrit values which include the buffy coat are read to ¼ of a division and averaged ($H'crit_0$). Two of these tubes are labeled "1" and the other two "2." The plasma is then drawn off all the tubes to a level 5 divisions above the surface of the buffy coat and replaced by Solutions A and B whose concentrations were decided upon empirically. The plasma removed from tubes numbered "1" is replaced by Solution A and the plasma removed from tubes numbered "2" is replaced by Solution B. All tubes are recapped and repeatedly inverted by hand until the erythrocytes are resuspended. They are then allowed to stand for thirty minutes to enable osmotic forces to reach an equilibrium. After this period the tubes are again centrifuged for thirty minutes at 3,000 r.p.m. and then the hematocrit values which include the buffy coats are again recorded. Hematocrit values of the two tubes numbered "1" are averaged to give $H'crit_1$ and the hematocrit values of the two tubes numbered "2" are averaged to give $H'crit_2$. $H'crit_0$ = average of initial hematocrit values. The value $Y = \frac{H'crit_0 - H'crit_1}{H'crit_2 - H'crit_1}$ is calculated. The plasma sodium in meq./L. is determined by finding the Y value of the blood sample in question and locating the point where this value intersects the curve in Fig. 1. Example: $H'crit_0 = 40$; $H'crit_1 = 37$; $H'crit_2 = 43$. Substituting the previously mentioned values in the equation for Y: $Y = \frac{40 - 37}{43 - 37} = 0.5$. The point where $Y = 0.5$ intersects the curve in Fig. 1 is located. Reading down from this point it is seen that the plasma sodium = 122

*4 ml. H'crit tubes calibrated 0-100. McAllister Bicknell Co., New Haven, Conn.

†Needle tube stoppers No. 500-C. Glasco Products Co., Chicago, Ill.

‡Heparin sodium 10,000 U.S.P. units per ml. Upjohn Co., Kalamazoo, Mich. (Sodium content in heparin solution added to each blood bottle = approximately .01 meq.)

meq./L. The curve shown may be reconstructed for more accurate reading on 10 × 10 to the inch graph paper using the following coordinates: Y = 0.909, X = 100; Y = 0, X = 148.5; Y = −0.3, X = 164.5. Hematocrit values throughout any single determination should be read by the same individual and read to ¼ of a division.

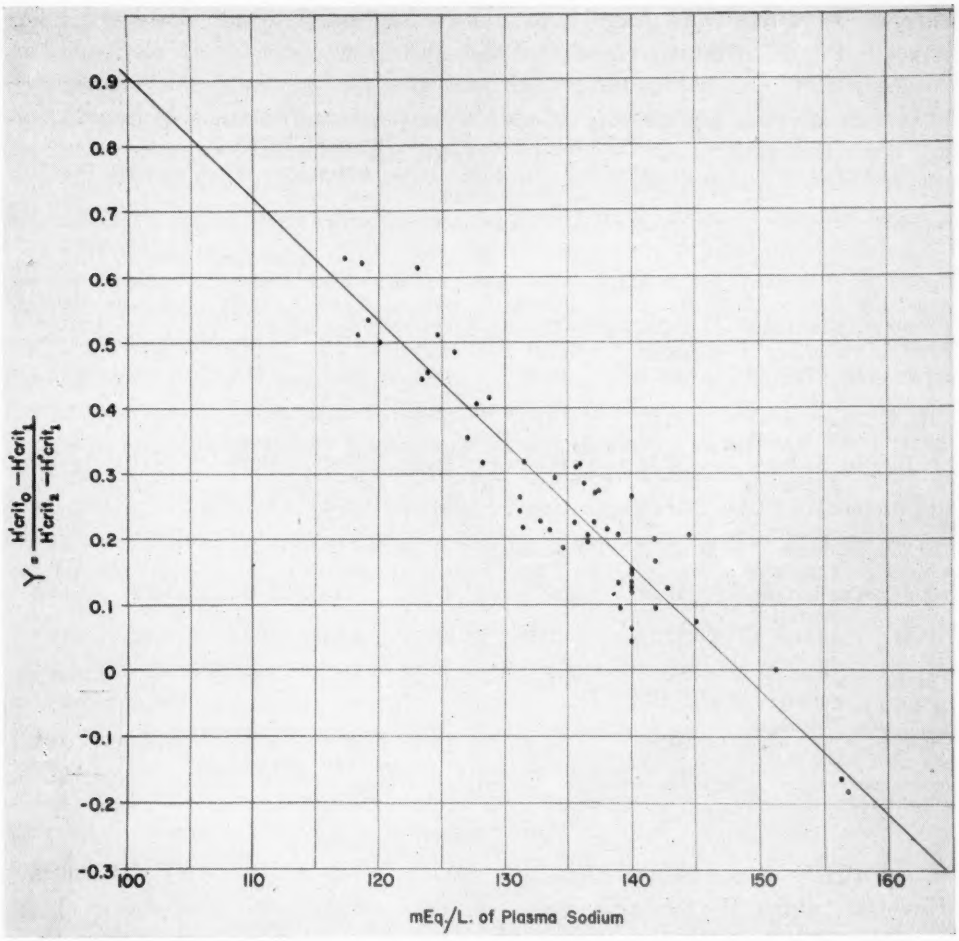

Fig. 1.—Relationship of Y value to plasma sodium concentration as determined by flame photometer. Regression line is superimposed.

RESULTS

Using the method as described, the Y values were obtained from the blood of 52 consecutive patients. Utilizing the plasma removed from the initial hematocrits ($H'crit_o$), simultaneous plasma sodium determinations were done with an internal standard flame photometer (Fox).* These plasma sodium values were then plotted against the Y values obtained. (See Scattergram, Fig. 1.) The coefficient of correlation of the data so obtained is 0.94. One standard error of deviation from the regression line for the points shown is ± 3.2 meq./L. based on values obtained by the flame photometer. As seen

*Built by Janke & Company, Inc., Hackensack, N. J.

from the data presented, there appears to be a straight-line relationship between erythrocyte volume and the plasma sodium concentration within the range of abnormal sodium values found in this study. Table I shows data obtained from 10 consecutive patients who were not included in the above series. Y values were found and plasma sodium determined by using the curve in Fig. 1 before it was reduced for publication.

TABLE I. COMPARISON OF PLASMA SODIUM VALUES OBTAINED BY METHOD DESCRIBED WITH THOSE OBTAINED BY FLAME PHOTOMETER

(The patients in this group were not included in the series from which curve in Fig. 1 was derived.)

PATIENT	DIAGNOSIS	$H'CRIT_0$ (AVERAGE)	$H'CRIT_1$ (AVERAGE)	$H'CRIT_2$ (AVERAGE)	(Y) VALUE	NA MEQ./L. FROM CURVE FIG. 1	NA MEQ./L. FLAME PHOTOMETER	DEVIATION IN MEQ./L.
H. C.	Tetanus	36.25	35.50	40.50	0.150	140.5	143.0	−2.5
J. P.	HCVD with CHF	42.75	41.50	47.25	0.217	137.0	135.0	+2.0
H. W.	Duod. ulcer N. and V.	53.25	52.00	58.00	0.208	137.5	140.0	−2.5
S. B.	L. Nephron Nephrosis	33.31	31.13	36.38	0.415	126.5	124.0	+2.5
M. A.	M. Myeloma Uremia	22.13	20.00	24.13	0.515	121.0	117.6	+3.4
H. J.	Diarrhea Etiology?	37.75	36.25	41.68	0.276	133.7	132.4	+1.3
J. J.	Ca. Larynx Postop.	36.00	35.00	40.13	0.195	138.1	137.6	+0.5
C. M.	Cirrhosis Ascites	37.75	34.50	40.00	0.591	117.0	117.6	−0.6
M. S.	Colostomy Postop. Diarrhea	39.00	37.00	42.50	0.364	129.2	125.4	+3.8
R. C.	R.H.D. with CHF	47.31	46.25	52.13	0.180	138.8	135.2	+3.6

DISCUSSION

Actually, it is assumed that total base is being measured by this method. However, since the osmotic value of the extracellular fluid rests almost entirely on sodium and since possible changes in the small concentrations of the other cations present would affect the total osmotic value only slightly, it can be assumed for all practical purposes, as noted by our findings, that erythrocyte volume varies inversely with the plasma sodium concentration. This relationship held in all cases examined. A variety of other electrolyte abnormalities were noted in this series beside variations in plasma sodium. These did not appear to have a noticeable effect on the results. No evidence of hemolysis was seen. The only trouble encountered in carrying out the procedure was difficulty in reading the hematocrits in two cases where the buffy coats were increased. As mentioned previously from Guest's work[5] one could expect similar results in blood dyscrasias where abnormal erythrocytes are present. An exception would be congenital hemolytic anemia where cells would hemolyze when mixed with a hypotonic solution such as "Solution B."

Other implications can be drawn from this study: (1) It can be assumed that the states of hyponatremia and hypernatremia found in this series were present for variable periods of time; yet the Y values determined were consistent with the plasma sodium at the time of measurement. In a patient with congestive heart failure whose plasma sodium remained between 119.2 milliequivalents per liter and 126.0 milliequivalents per liter for a period of twenty-five days as found by frequent flame photometer determinations, the Y value at the beginning and end of this period showed it to be consistent with the patient's plasma sodium measured at those times. This may mean that the osmotic forces within the erythrocyte in vivo remain constant over a long period of time and do not change in response to alterations of osmotic value in the extracellular fluid. (2) In determinations which depend on the integrity of the hematocrit, such as mean corpuscular volume, mean corpuscular hemoglobin concentration and erythrocyte potassium, hyponatremia and hypernatremia introduce an additional source of error because of the changes in cell volume. (3) Does the increase in cell volume as noted here with hyponatremia cause increased fragility of erythrocytes and account in part for the anemia seen in chronic uremia, acute renal shutdown, and other syndromes where a low plasma sodium is frequently noted? (4) The increase in hematocrit which is seen in dehydration associated with a low plasma sodium such as severe diarrhea, vomiting, burns, etc., is due in part to the previously mentioned phenomenon and not due to hemoconcentration alone.

SUMMARY

A simple, indirect method to determine plasma sodium is described. It consists mainly of reading the initial hematocrits, then substituting two known solutions for part of the plasma, and finally redetermining the new hematocrit values. This procedure can be carried out in any small laboratory where hematocrit tubes and a suitable centrifuge are available. The method is considered accurate within the limits necessary for clinical purposes.

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A SMALL DRYING APPARATUS DESIGNED FOR PRESERVATION OF VIRUSES

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DURING the conduct of virus research, it is often desirable to desiccate small amounts of valuable infected material at short notice.

In such circumstances, one is reluctantly compelled to postpone the work until such time as a sufficient volume of material has been accumulated to justify the expenditure of time, energy, and Dry Ice in undertaking the operation.

Thus, there is need for a small, easily operated, high efficiency vacuum drying chamber for drying viruses with minimum loss of potency and a low consumption of Dry Ice. The instrument described has been specifically designed with the main objective of the retention of potency of infective material during the drying process, rather than toward the achievement of mere speed in drying.

Construction.—With the exception of two detachable metal disk lids, the entire chamber is welded together as a unit, constructed from brass tubing, and weighing 35 pounds. All joints may be soldered with lead or better still, electrically spot welded. The completed chamber should be carefully tested and should be proved to be vacuum tight.

Fig. 1 and accompanying legend reveal all structural details and are self-explanatory. The instrument has been built in such a manner as to minimize the possibility of vacuum leaks. The assembled equipment is shown in Fig. 2.

PREPARATION OF EQUIPMENT

Sterilization of Drying Chamber.—Pieces of heavy wall pressure rubber tubing are fitted to each metal nozzle (Fig. 1, *W*), and the open ends are wrapped in kraft brown paper and secured with rubber bands. Thereafter, the complete apparatus is wrapped with kraft brown paper, enclosed in a carton box and sterilized by autoclaving for one hour at 10 pounds pressure per square inch (250° F.).

Preparation of Desiccant.—Two wire baskets are filled with anhydrous $CaCl_2$ or Dessigel drying agent and heated in the hot air oven at 115° C. for three hours.

Assembly of Apparatus.—After sterilization, the cylinder, (*H*, Figs. 3 and 4) is placed on the supporting tripod frame (*J*); Castaloy metal hose cocks, (*K*) are applied to each piece of rubber hose (*L*) and are firmly screwed against each piece of tubing.

Thereafter, both metal lids (*D*, Fig. 3) are removed from the top of the cylinder, and 2 baskets containing dry calcium chloride (*T*, Fig. 1) are removed from the hot air oven

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This work has been undertaken with the aid of Grant No. 382 from The Defence Research Board, Ottawa, Canada.

Received for publication, Nov. 6, 1953.

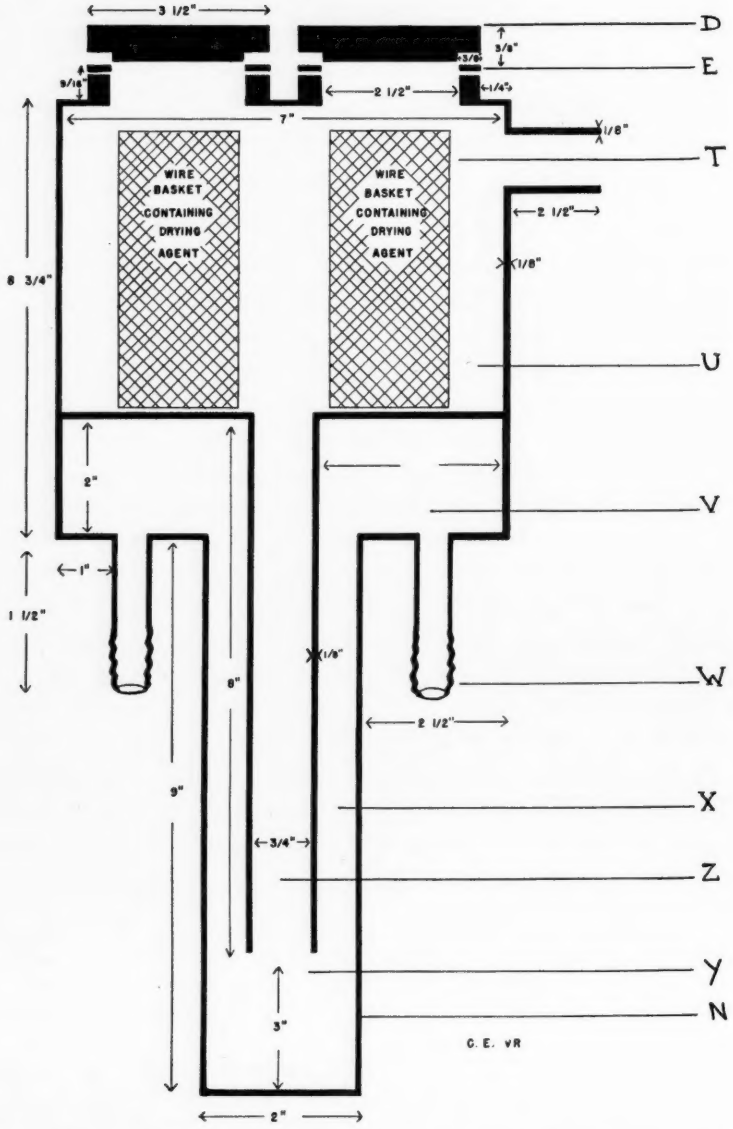

Fig. 1.—Showing vertical section diagram of drying chamber indicating size, dimensions and design. Moisture vapor from material being dried, enters through nozzle. (*WEN*), passes upward into the first chamber *V*, thereafter it is drawn downward into the vapor chamber (*X*) and frozen at space (*Y*). Residual moisture vapor next moves upward along the lumen of central tubing (*Z*) to enter the third chamber above (*U*), containing $CaCl_2$ drying agent (*T*). Other components are: (*D*) metal lid (*2*), neoprene rubber gasket (*E*); and wire gasket containing drying agent; lower end of vapor trap.

and immediately inserted within the chamber (*U*, Fig. 1). The rubber gaskets (*E*, Figs. 1 and 3) are promptly sealed with Celloseal cement and clamped down with the aid of the cross bar (*C*) and wing nuts (*B*). The chamber is now connected to the Cenco Megavac vacuum pump which should be operated for one hour during which the system should be checked for leaks with the aid of a McLeod gauge (*Q*, Fig. 5). Preparatory to proceeding with any attempt to dry material, a vacuum of at least 100 microns of mercury should be attained. Assuming that the chamber has been properly constructed, failure to reach 100 microns may indicate a leaking rubber gasket seal, a faulty Castaloy clamp, an inefficient pump, the use of an incorrect grade of vacuum pump oil or oil containing water. Fresh oil should be used for each new run.

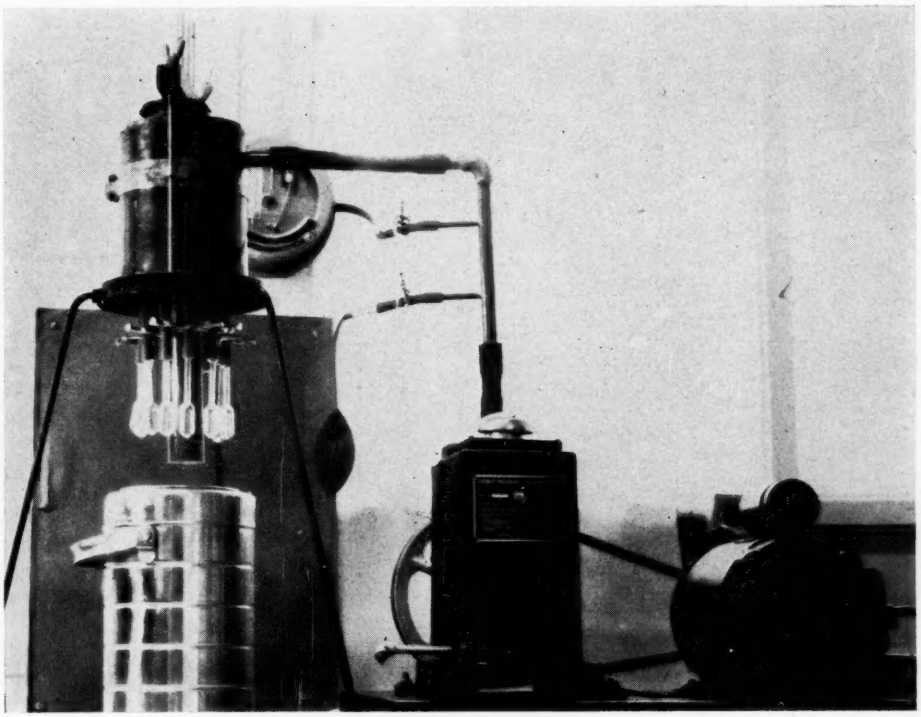

Fig. 2.—Photograph showing complete apparatus, consisting of drying chamber, Cenco Megavac vacuum and pump and electric motor.

METHOD OF OPERATION

Preparation of Virus Material for Drying.—Two milliliter volumes of finely divided infected tissue are placed in each of 12 glass ampules (*M*, Fig. 4) of 10 ml. capacity and shell frozen by quick manual rotation in a mixture of CO_2 and alcohol at −72° C.

Subsequently, each ampule containing frozen material is inserted by forcing it into each rubber connection tube to a depth of 1 inch and simultaneously, the attached pinchcock is promptly released and discarded. This procedure is repeated until twelve ampules are placed in position, and each of the twelve pinchcock clamps have been removed. To obviate sudden rises of pressure within the chamber, ampules are inserted at three-minute intervals to allow sufficient time for reduction of vacuum to the level requisite for retaining material in the frozen state.

Operating Details.—Having set all 12 ampules in their respective positions, the latter are duly inspected to ensure that each ampule is covered with white frost. Provided that this

is so, then satisfactory drying may be anticipated. All being well, the CO_2 alcohol cooling mixture is now prepared by placing crushed Dry Ice to a depth of 4 inches at the bottom of the stainless steel vacuum jar (*O*, Figs. 4 and 5).

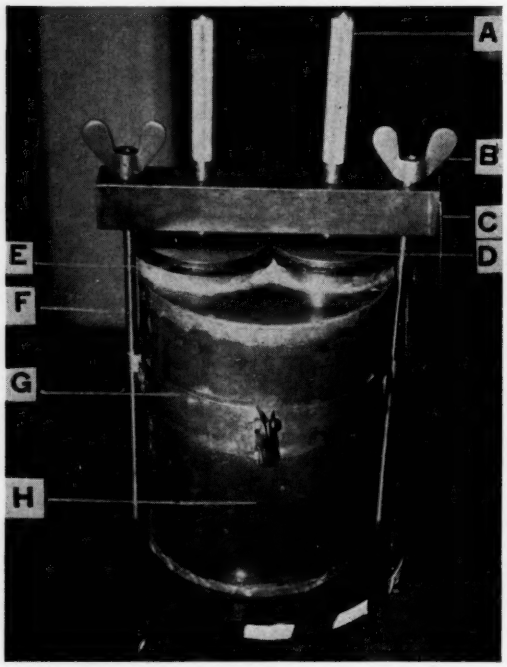

Fig. 3.

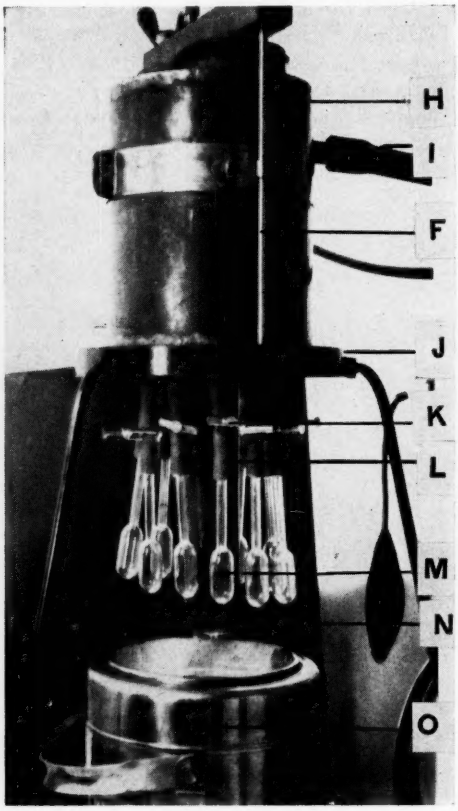

Fig. 4.

Fig. 3.—Showing upper end of drying chamber and method of application of mechanical pressure to metal lids (*D*). Components are as follows: (*A*) adjustable centering pin (2); (*B*) wing nut (2); (*E*) neoprene gasket, (*F*) upright metal post (2) for support of cross bar (*C*) and application of counterpressure exerted by centering pins (*A*) and wing nuts (*B*); (*G*) metal band for support of upright posts (*F*); (*H*) outside surface of drying chamber.

Fig. 4.—Showing (*H*) drying chamber, (*I*) wide, 1 inch diameter rubber hose to vacuum pump, (*F*) upright support posts. (*J*) tripod head, (*K*) Castaloy pinchcock (12). (*L*) pressure rubber hose (12), (*M*) ampule (12), (*N*) lower end of vapor trap (see also Fig. 1), (*O*) vacuum flask.

To facilitate insertion of the vapor trap (*N*, Figs. 1 and 4) into the Dry Ice alcohol mixture, a cylindrical roll of wire gauze was placed in the center of the vacuum flask before the Dry Ice was packed around the foot of the gauze. The latter could not be shown in the photograph. Absolute alcohol is now added slowly to the vacuum flask and the jar raised by inserting beneath it a wooden block 9¾ by 9½ by 8 inches (*P*, Fig. 5), so as to immerse the foot of the vapor trap and glass ampules in freezing mixture.

The position of the equipment of operation is shown in Fig. 5. Dry Ice is replenished as it melts and approximately 1 pound of powdered material is added every four to six hours. When employing a total fluid capacity of 24 ml., drying is usually complete in about twenty-four to thirty-six hours. The preservation of a high degree of potency in infective material is considered to be of greater importance than speed of drying.

Method of Changing $CaCl_2$ During Run.—One advantage of the equipment described is that wet $CaCl_2$ may be replaced by freshly dried material at any time during the run while material is still held under vacuum in the frozen state. In practice we have found it advantageous to change the calcium chloride after twelve hours of continuous drying.

This is accomplished as follows: replace hose cocks on rubber tubing fitted to each ampule while the latter continues to be immersed in freezing mixture as previously. Thereafter, the motor is switched off; the vacuum released through the bleeder connection (*R*, Fig. 5) the two clamping bolt wing nuts (*B*) and metal lids are removed, the wet $CaCl_2$ removed and two baskets of freshly dried $CaCl_2$ are substituted. The metal lids are replaced again, clamped down, and the motor switched on. After the vacuum has reached 100 microns, or less, at intervals of three minutes, each hose cock is released in turn and removed as previously.

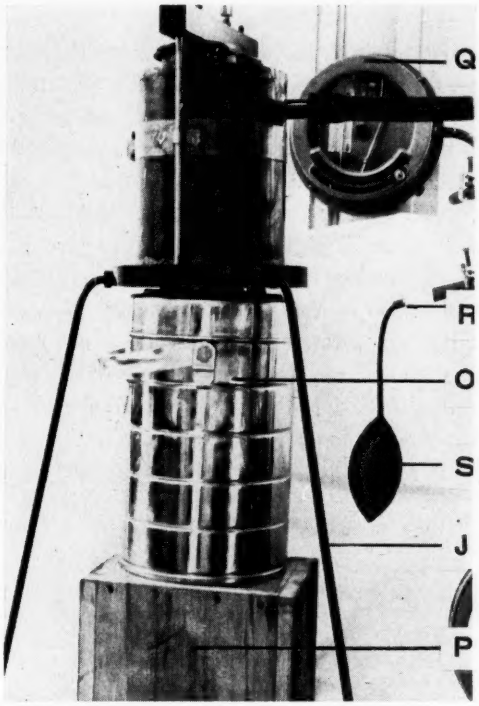

Fig. 5.—Showing view of apparatus in the operating position. The vacuum flask (*O*) has been raised with the wooden block. (*P*) and ampules and vapor trap have been immersed in freezing mixture. Other parts are: (*J*) tripod leg (3), (*S*) rubber gas bag, (*R*) detachable bleeder point, (*Q*) vacuum pressure gauge.

Sealing Off Ampules.—After drying is complete, the vacuum flask is removed, the motor is stopped, a cylinder of dry nitrogen gas is connected with rubber hose to the bleeder opening and the vacuum replaced with nitrogen gas. The optimum pressure of nitrogen may conveniently be selected by attaching a rubber gas bag (*S*, Fig. 5) to the system, so as to admit sufficient positive pressure, enough to expand the bag slightly. Finally, each ampule is sealed off at the neck with an oxypropane flame.

Advantages claimed are as follows: (1) Everything may be sterilized by autoclaving. (2) From the start to finish, ampules containing virus material are immersed in Dry Ice and alcohol freezing mixture. (3) To accelerate drying, the vacuum pump may be stopped, the chamber opened, and freshly dried

$CaCl_2$ substituted, while ampules are immersed in freezing mixture and the material within them, still held under vacuum. (4) Likewise, if necessary, vacuum pump oil may be changed in the middle of a run while ampules and contents continue to be retained in freezing mixture under vacuum. (5) A single supply of CO_2 Dry Ice mixture provides the necessary freezing agent for the dual function of operating the moisture trap as well as maintaining ampules at –72° C. throughout the drying process. (6) When drying is complete, the vacuum may be replaced with dry nitrogen gas and the ampules sealed off with blow torch under atmospheric pressure. (7) As nitrogen gas is allowed to enter the system, traces of moisture present in the gas are automatically removed by the calcium chloride drying agent and freezing mixture.

THE ESTIMATION OF PROFIBRINOLYSIN

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INTRODUCTION

IN REVIEWING the literature concerning the measurement of plasma proteolytic enzyme active at pH 7.8, the comparison and correlation of results become confusing owing to the variety of techniques used and the units of activity adopted by different workers. While the method about to be described is similar to existing techniques, its chief advantage is that with it one can obtain proportionate recovery of proteolytic activity at different dilution levels of plasma. Since Loomis' preparation "Fibrinolysin"[4]† has been used in the preparation of the standard curve, his unit system has been adhered to in expressing the unitage in plasma. The technique is simple, requiring only ordinary skill and care and no expensive or unusual equipment is necessary.

The present report is concerned with a method for the determination of total available proteolytic activity (at pH 7.8) in human plasma. This, theoretically, includes both profibrinolysin and fibrinolysin. Most of the work so far has been done on normal specimens from which the latter is absent.

MATERIALS AND METHODS

Assay of Fibrinolytic Activity.—In the present study the measurement of the lysis time of a standard fibrin clot has been used to measure the proteolytic activity of, first, the preparation of bovine fibrinolysin and, second, of the activated human profibrinolysin. This assay is very similar to those used by Westphal and co-workers,[9] Guest, Ware, and Seegers,[2] and Lewis and Ferguson.[3]

Materials for Assay.—

Borate Buffer, pH 7.8.

Fibrinogen, Armour Bovine Plasma Proteins Fraction I (60.55 per cent clottable by weight); 0.5 per cent solution in borate buffer. (In later work 0.3 per cent fibrinogen has been used.) This solution is made up in quantity and 0.5 ml. aliquots are measured into flanged culture tubes. Those which are not being used within three hours are covered securely with Pliofilm and kept frozen at –10° C. until immediately prior to use.

Thrombin, Parke, Davis Topical Thrombin; 10 units/ml. in borate buffer. (This is also stored frozen in aliquots suitable for one day's assay.)

From the McGill University Clinic, Royal Victoria Hospital, Montreal.
Aided by a grant from the Canadian National Research Council.
Received for publication, Nov. 15, 1953.

*This work was done during the tenure of a Graduate Medical Research Fellowship granted by the National Research Council of Canada.

†Supplied through the courtesy of Dr. E. C. Loomis, Parke, Davis & Company, Detroit, Mich.

Fibrinolysin, Bovine fibrinolysin. This is dissolved in borate buffer in concentrations from 0.5 to 3.0 units/ml. and used 20 to 60 minutes after solution. Assay is carried out as follows:

To 0.5 ml. of 0.5 per cent (or 0.3 per cent) fibrinogen solution is added 1 ml. of enzyme solution, or solution being tested for enzyme content. A stop watch is started at the same moment that the enzyme solution is allowed to start flowing; 0.2 ml. of thrombin solution (10 units/ml.) is then run in from a burette and the tube is gently shaken until the watch shows that 30 seconds have elapsed since the enzyme was first added. The tube is then placed in a Pyrex constant-temperature water bath at 37° C. and observed without further manipulation. Bubbles of air become trapped in the clot as it forms and rise to the meniscus at the time of dissolution as a result of the proteolytic action of the fibrinolysin. The watch is stopped as the fifth bubble reaches the surface. This time, to the nearest second, is the lysis time of the standard fibrin clot.

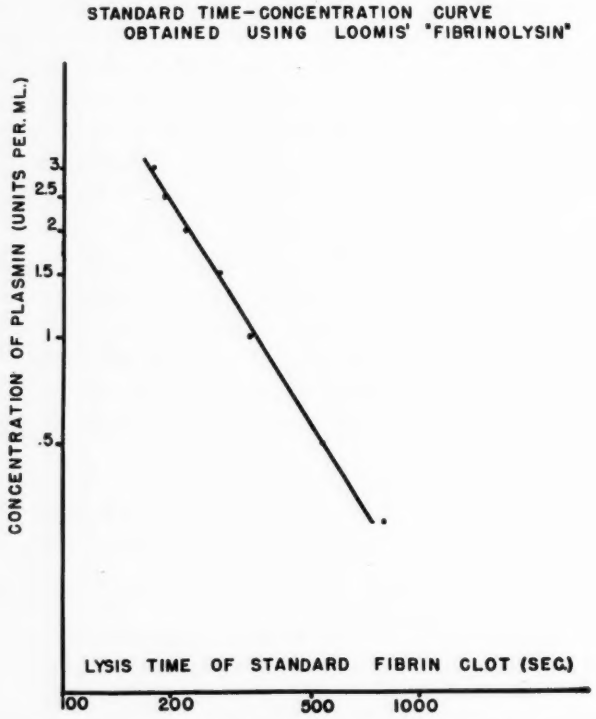

Fig. 1.—The concentration of fibrinolysin is referred to as plasmin in this figure.

Fig. 1 shows the inverse logarithmic relationship between the concentration of fibrinolysin (expressed in terms of Loomis' units per milliliter) and the lysis time of the standard fibrin clot. This graph represents the average values obtained on four different occasions from four different vials of the most potent batch of enzyme yet received. Variation between values obtained at different assays for the same point on these curves was never greater than thirty seconds.

Lederle's Streptokinase-streptodornase* dissolved in borate buffer was used to activate human profibrinolysin prior to assay as outlined previously. When otherwise untreated dilutions of plasma or serum were used as the source of profibrinolysin, quantitative recovery of

*Supplied through the courtesy of Dr. J. M. Ruegsegger, Lederle Laboratories Division American Cyanamid Company, Pearl River, N. Y.

proteolytic activity was inconsistent. The range of values was similar to that obtained on the ammonium sulfate precipitate, but the latter always ran 100 to 200 units/ml. higher. It was evident, therefore, that some further separation of profibrinolysin from plasma was necessary for quantitative estimation.

Different techniques such as isoelectric precipitation as carried out by Ungar,[8] acidification of plasma as done by Cliffton,[1] and the alcohol fractionation technique of Richert[5] all left considerable profibrinolysin capable of activation in the supernatant fluid. In the first instance values ran as high as 0.94 unit/ml., the second about 0.5 unit/ml. and in the last-mentioned method of precipitation the supernatant fluid contained 0.25-0.3 unit profibrinolysin per milliliter. After much trial and error, precipitation with half-saturated ammonium sulfate of plasma dilutions at pH 3.7 was found to give the most satisfactory results. This technique provides a complete precipitation of profibrinolysin and allows for its estimation at higher original dilutions of plasma than is possible with any of the other precipitation techniques tried. Within a reasonable range ($\frac{1}{250}$ to $\frac{1}{1,000}$) satisfactory recovery of activity at different dilution levels is obtained.

Precipitation of Profibrinolysin.—
Materials:

Acetate Buffer, pH 3.7 (185 ml. N/10 acetic acid to 15 ml. n/10 sodium acetate. Adjusted to pH 3.7).

Borate Buffer, pH 7.8.

Ammonium Sulfate, Saturated solution in distilled water at 5° C.

Cell-free plasma is diluted with acetate buffer (pH 3.7) in volumes varying from one part plasma to 250 parts buffer up to one part plasma to 1,000 parts buffer, and mixed thoroughly. Two milliliters of the diluted plasma are placed in a centrifuge tube and kept cold during the precipitation procedure. Two milliliters of cold, saturated ammonium sulfate are then added very slowly, with constant shaking of the tube. The tubes are then covered with Pliofilm and kept at 5° C. overnight (sixteen hours). Shorter periods of precipitation decrease the yield of enzyme, with less satisfactory duplication of results from identical samples.

Centrifugation is then carried out in a high-speed refrigerated centrifuge for three hours at 18,000 r.p.m. The supernatant fluid is decanted immediately and the precipitate dried in a desiccator over calcium chloride at 5° C. Any specimens which are not assayed at this point are covered and stored in a deep-freeze unit in the dry state.

Activation of Profibrinolysin.—Maximum activation of profibrinolysin seems to occur when the final concentration of streptokinase is 1,111 units per milliliter. This is obtained by dissolving the ammonium sulfate precipitate in 4 ml. of borate buffer at the time of assay, and adding 0.5 ml. of streptokinase solution (10,000 units per milliliter). The contents of each tube are dissolved and mixed by at least 20 inversions. Maximum activation takes place in thirty minutes at 37° C. with no significant change in activity if the specimens are kept for another thirty minutes at room temperature.

RESULTS

No grossly detectable fibrinogen could be found in the ammonium sulfate precipitate, nor was there any antifibrinolysin activity. The supernatant fluid was tested for profibrinolysin after dialysis against cold distilled water, followed by partial evaporation in the cold in order to concentrate it, and subsequent activation by streptokinase. When precipitation is carried out as described previously, there is no loss of profibrinolysin to the supernatant.

Streptokinase alone takes forty-eight hours to dissolve the clot. This is probably due to contamination of commercial fibrinogen with profibrinolysin.

TABLE I. SHOWING RECOVERY OF PROFIBRINOLYSIN FROM DIFFERENT DILUTIONS OF PLASMA (All samples are from normal individuals. Results are expressed as Loomis' units fibrinolysin per milliliter of undiluted plasma)

SOURCE	SEX	DILUTION	LYSIS TIME OF CLOT (SEC.) (AVERAGE OF TWO DETERMINATIONS)	UNITS PROFIBRINOLYSIN PER ML. TEST	UNITS PROFIBRINOLYSIN PER ML. PLASMA
C. G.	M	1/250	285	1.44	810
		1/500	435	0.7	787
		1/1,000	632	0.39	875
R. S.	F	1/400	420	0.74	666
		1/500	460	0.64	720
		1/1,000	710	0.32	720
D. C.	M	1/400	485	0.592	532
		1/500	519.7	0.532	597
		1/800	750	0.3	540
M. L.	M	1/400	373.5	0.91	819
		1/500	434.5	0.71	800
		1/1,000	675	0.35	790
F. L.	M	1/250	312	1.16	654
		1/400	402	0.795	715
		1/500	472	0.62	700
J. A.	M	1/250	315.5	1.17	659
		1/500	495	0.57	640
A. C.	M	1/400	370.7	0.9	809
		1/500	407	0.78	876
B. F.	F	1/400	468	0.63	567
		1/500	539.5	0.5	562
J. M.*	M	1/400	484	0.5	450
		1/500	516	0.42	472
		1/800	601.5	0.277	496
B. S.*	F	1/400	469	0.54	486
		1/500	548	0.352	398
		1/800	645	0.23	414

*The results are calculated from dilution curves using 0.3 per cent fibrinogen as substrate

The error is negligible and constant in all determinations. The globulin precipitate alone has never shown spontaneous proteolytic activity in the dilutions employed.

In Table I are presented the results obtained when different dilutions of plasma are treated in the manner described. Concentrations of plasma above one part in 250 give a decreased proportionate yield of activity. Thirty seconds is the maximum acceptable difference between duplicate determinations. In an active preparation this may mean a difference of 0.2 unit per milliliter which, when multiplied by the plasma dilution factors, may cause a rather large experimental error. For this reason it is preferable to work at dilutions where the readings are at least 350 seconds.

Further error is introduced if the lysis time takes longer than 600 seconds, since the more prolonged the lysis time, the slower and more indefinite is the exact moment of dissolution of the clot. Also, the straight-line relationship of

the standard curve falls off at enzyme concentrations below 0.35 unit fibrinolysin per milliliter. For routine use a dilution of 1/400 (0.05 ml. plasma/20 ml. buffer) is used.

The number of units of fibrinolysin per milliliter is obtained from the standard curve (Fig. 1). This value is then multiplied by the appropriate dilution factors (½ the original plasma dilution × 4.5) to give the number of units of profibrinolysin per milliliter of original undiluted plasma.

From Table I it can be seen that the units of profibrinolysin per milliliter are approximately proportional to the concentration of plasma employed, indicating a quantitative recovery of profibrinolysin activity.

DISCUSSION

The method as outlined for the determination of total available proteolytic activity of plasma certainly does not fulfill all the criteria of an accurate biochemical determination. It does, however, present very definite advantages over previously described techniques. The separation of profibrinolysin is complete and, although many other substances are undoubtedly precipitated with it, they do not seem to interfere significantly with proportional recovery of activity within the dilution range used. The range is such that one should be able to assess quantitatively a wide variation of profibrinolysin level, if such occurs. The normal levels so far obtained have fallen between 400-900 Loomis units per milliliter. Specimens taken after strenuous muscular exercise, in a patient with acromegaly and another with hemophilia, have not been outside the normal range.

The assay of proteolytic enzymes by means of fibrinogen tagged with radioactive iodine[6, 7] is probably much more sensitive than is the lysis time of a standard fibrin clot. Under these circumstances even higher dilutions of plasma may be used for the estimation of profibrinolysin than has been possible in our experiments. Greater dilution may obviate the necessity of precipitation by rendering less significant whatever substances interfered with quantitative recovery of profibrinolysin from untreated plasma. Any method, however, using radiactive elements involves the use of special equipment and techniques not generally available at present, while the method described here requires no unusual apparatus or specially trained personnel.

SUMMARY

A method for the quantitative estimation of profibrinolysin is described in which profibrinolysin is precipitated with half saturated ammonium sulfate, activated with streptokinase and assayed by determination of the lysis time of a standard fibrin clot.

The precipitation is complete. No profibrinolysin is lost to the supernatant fluid.

Quantitative recovery of profibrinolysin is obtained from dilutions of plasma varying from $\frac{1}{250}$ to $\frac{1}{1,000}$.

Normal profibrinolysin levels in human subjects fall between 400 and 900 Loomis units per milliliter plasma.

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